IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Attila T. Lorincz, et al.	Group Art Unit: 1634
Serial No	:	09/970,477	Examiner: Johannsen, Diana B.
Filed	:	October 4, 2001	
For	:	ASSESSMENT OF HUMAN PAPILLOMA VIRUS-RELATED DISEASE	

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is a Declaration under 37 C.F.R. §1.132 by Dr. Attila T. Lorincz in the above-identified application.

I, the undersigned, Attila T. Lorincz, declare and state that:

1. I am a co-inventor of the subject patent application having serial no. 09/970,477.

2. My education and professional experience as an expert in the area of nucleic acid chemistry and analysis are set forth on the attached copy of my Curriculum Vitae.

3. As stated on my Curriculum Vitae attached herewith, my area of expert training and experience is in nucleic acid chemistry, in the analysis of nucleic acids in biological samples, and in the use of such nucleic acid analyses to develop diagnoses and prognoses concerning diseases related to the organism from which the nucleic acid was obtained.

4. I have read and understand the February 13, 2003 and April 19, 2004 Official Actions issued in the above-identified case. In particular, I understand that the Examiner has rejected claims 8-12 because the Examiner contends that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to

make and use the invention commensurate in scope with these claims. As a person skilled in the art, I respectfully disagree with the Examiner's rejection.

5. As shown by the evidence below, there is a strong and universally accepted correlation between the cell culture models of the specification and diseases in patients. The teaching of the application can be used by the skilled practitioner for assessment and diagnosis of disease stage in cells, including patient cells. For the experiments described, *in vitro* results are generally correlative with *in vivo* results. Moreover, *in vitro* data are routinely used by those in the art who apply and extrapolate the findings and outcomes of *in vitro* cell culture experiments to *in vivo* use.

6. Infection with human papillomavirus (HPV) has been confirmed to be the cause of virtually all cases of cervical cancer (*see*, for example, Walboomers, et al., *J. Pathol.* 189:12, 1999; **Exhibit 1**) and has been identified in a high percentage of non-melanoma skin cancers, in cancers of the oral cavity, the larynx, and the esophagus, in intraepithelial neoplasias and in various type of hyperkeratoses (*see*, for example, zur Hausen, *Biochimica et Biophysica Acta* 1288:F55, 1996; **Exhibit 2**). In fact, HPV is associated with a spectrum of lesions ranging from benign epithelial hyperproliferation to invasive carcinomas. For example, viral gene expression of HPV is tightly linked to cellular differentiation. (*See* Broker et al. *Cancer Cells*, 7:197, 1989; **Exhibit 3**). *See* also Southern and Herrington (*Sex Transm. Inf.* 74:101, 1998; **Exhibit 4**) who describe that human papillomavirus has been identified as the major aetiological factor in cervical carcinogenesis. In addition, Stoler (*Int. J. Gynecol. Pathol.* 19:16, 2000; **Exhibit 5**) describes human papillomaviruses as the etiologic agents of cervical neoplasia.

7. The zur Hausen publication describes HPV types 1 through 70 and the human pathology related to infection with each type of HPV. The Walboomers publication describes the worldwide HPV prevalence in 99.7% of cervical carcinomas.

8. However, most human papillomavirus infections resolve spontaneously without causing cancer or development of cancer precursors. Therefore, it is useful to be able to distinguish those infections that may progress to cancer from those that may resolve spontaneously. Furthermore, it is useful to employ treatments early that may be effective in preventing and/or treating the human papillomavirus infection in the pre-cancerous stage so that the progression to cancer can be inhibited.

9. Generally, human papillomaviruses cannot be cultured *in vitro*, although HPV type 16 has been cultured *in vitro* with limited success. Therefore, model systems for human patients have been developed to study the stages of HPV infection and their relationship to the development of HPV-induced neoplasia and cancer.

10. Cell lines such as HaCaT, W12, and SiHa are universally recognized by researchers as model systems of human epithelium in a patient at various stages progressing to cancer. These human cell lines contain different HPV copy numbers per cell and are used to model different stages of HPV-induced neoplasia and cancer in human patients. For example, the HPV negative cell line, HaCaT, is an immortalized cell line that is useful as a model system. Cultures of HaCaT cells, transfected by HPV 16 DNA that are in the episomal form, represent a useful model of early HPV infection typical of a non-neoplastic lesion. Cell lines that contain an increased amount of HPV in an episomal form, such as W12, represent a pre-malignant condition in humans. Cell lines that contain HPV in an integrated form, such as SiHa, represent relatively advanced human cancer.

11. The HaCaT cell line is a naturally immortalized human keratinocyte cell line similar in some ways to W12. (*See* Boukamp et al., *J. Cell Biol.*, 106:761, 1988. **Exhibit 6**). HaCaT cells correlate to human epithelium, in particular, keratinocytes, and are used to model human disease in a patient. Thus, HaCaT cells infected with HPV represent human patient epithelium infected with HPV. The Boukamp article describes HaCaT as the first permanent epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for studying regulation of keratinization in human cells. The cell line maintains full epidermal differentiation capacity and remains nontumorigenic even when transplanted onto nude mice. (*See* Abstract). The Boukamp article concludes that "the HaCaT cell line is closely approximated to normal keratinocytes, and thus offers a suitable model to study regulatory mechanisms in the process of differentiation of human epidermal cells....This cell line provides a valuable model system for the study of the role of oncogenes and other factors in the process of malignant conversion of human epithelial cells." (*See* page 770, last paragraph).

12. White et al. (*J. Virology*, 72:959, 1998; **Exhibit 7**) describes the infection of HaCaT cells with HPV virions extracted from HPV-infected human condylomas. The HPV-infected HaCaT cells were xenografted onto mice in order to test neutralizing antibodies prepared as part of an HPV vaccine development program. The White article states that

based on this model system, an *in vitro* assay which can be used to study the early stages of HPV-16 infection was developed. (*See* page 962, right hand column). Thus, this study further demonstrates the usefulness of the HaCaT cell line as a model for early stage HPV infection.

13. The W12 cell line, which contains HPV in an episomal form, correlates to human epithelium, in particular, keratinocytes, and is used to model premalignant human disease. Coleman, N. and Stanley, M. A.(*Hum. Pathol.* 25:73-79, 1994; **Exhibit 8**) describe the cervical keratinocyte cell line W12 as a model for low-grade squamous intraepithelial lesions and that the SiHa and CaSki cell lines are models for high-grade squamous intraepithelial lesions and cancers. (*See* Abstract).

14. Coleman and Stanley conclude that "differential expression patterns in cervical keratinocytes *in vivo* are mirrored by the *in vitro* finding that L1 is only patchily present in the high-grade epithelium produced by CaSki and SiHa cells, but is strongly expressed in differentiated layers of NCx [normal cervix] and W12 cells." (*See*, pg. 78, col. 1-2). Thus, Coleman and Stanley establish a strong correlation between the W12 and SiHa cell lines and human patients.

15. SiHa cells which contain HPV integrated into the genome correlate to human epithelium, in particular, keratinocytes, and are used to model malignant human disease. Rong, et al. (*Chinese Medical Journal* 109:854, 1996; **Exhibit 9**) describe the use of CaSki, SiHa, HeLa, and W12 cell lines in determining the susceptibility of HPV-infected keratinocytes to lysis by specific cells generated by the immune system. The authors describe the cell lines as follows:

16. "Cervical carcinoma derived keratinocytes, CaSki (HPV16+, 300 -500 copies), SiHa (HPV16+, 1 copy) and HeLa (HPV18+, 100 copies), were maintained in continuous culture in Glasgow's modification of Eagle's medium (GMEM) containing 10% FCS at 37 ^{o}C in a 5% CO_2 incubator. <u>They were used as models of high grade squamous intraepithelial lesion (HSIL)</u> according to the Bethesda system." (Rong, et al. Page 855, col. 1, 2nd complete par.; emphasis added).

17. "<u>W12, as a model of low grade squamous intraepithelial lesion (LSIL)</u>, is a cervical keratinocyte line which is immortalized but non-transformed by natural infection with HPV 16....It contain[s] about 100 copies of HPV 16 DNA in the episomal form." (Page 855, col. 1, 3rd complete par.; emphasis added).

18. Thus, Rong, et al. establish that cell lines such as CaSki, HeLa, HaCaT, W12, and SiHa are universally recognized by researchers as model systems of human patient epithelium at various stages progressing to cancer.

19. Tan and Ting, (*Cancer Research* 55:4599, 1995; **Exhibit 10**) correlate the response of SiHa and CaSki cell lines to the response of SiHa-induced human tumors in nude mice when treated with phosphorothioate oligonucleotides. HPV RNA was measured in CaSki cells and in the tumor cells extracted from the mice before and after treatment. The results demonstrated that treatment with the antisense oligonucleotides resulted in the reduction of HPV RNA expression levels in both the CaSki cells and the mouse tumors, in the inhibition of CaSki and SiHa cell proliferation, and in the inhibition of mouse tumor growth.

20. Madrigal, et al. (*Gynecol Oncol*, 64:18, 1997; **Exhibit 11**) report reducing the expression of the HPV oncogenes with phosphorothioate oligonucleotides in cervical cancer cells. In this publication, SiHa, CaSki, and HeLa cell lines were used as models of cervical cancer and the results were shown to be consistent with similar experiments that used cells cultured from primary cervical tumors. The IC_{50} data obtained from the SiHa and CaSki cells and the primary cervical tumor cells were virtually identical. These data demonstrate the usefulness of SiHa and CaSki cells lines as models for human cancer.

21. Additionally, Z. Naghashfar, et al. (*Cancer Letters* 100:47-54, 1996; **Exhibit 12**) describe prostate tumor progression models where HPV 16 DNA and HPV 18 DNA are used to immortalize human prostate epithelial (HPE) cells. The human prostate epithelial (HPE)/HPV immortalized cell lines are useful as a model for examining the effects of therapeutic agents for treating androgen-independent prostate tumor cells. Detection of HPV 16 and 18 sequences were confirmed with positive controls SiHa cell DNA and HeLa cell DNA, respectively. HeLa cells are human cervical carcinoma cells derived from keratinocytes and are useful as a model system for human disease.

22. Koromilas, et al. (*Cytokine & Growth Factor Reviews* 12:157-170, 2001; **Exhibit 13**) report that the HPV is the infectious agent in cervical neoplasia where the major risk factors are high risk HPV types 16, 18, 31, 33, 35, 39, and 41-45. The review also states that "the only viral genes which are consistently expressed following integration is the E6 and E7 oncogenes and these genes are critical for the development of malignant transformation and also play a role in altering the cellular response to cytokines" (page 158, col. 1, 2nd par.).

Koromilas, et al. associate the various HPV types and HPV genes, such as E6, E7, E5, E4, E2, E1, L1, and L2, to transformation and disease.

23. These are but a few examples of a wide body of literature that support the use of HPV-containing cell lines as models of human cancer and the widespread use of these cell lines to investigate potential preventive and therapeutic treatments for human cancer patients.

24. As shown by the evidence below, there is a correlation between HPV transcripts and various disease stages. For example, Mark H. Stoler (*Intl. J. Gyn. Path.* 19:16-28, 2000; **Exhibit 5**) supports the association of CIN1 and CIN3 or invasive squamous cancers with HPV types. Infection by HPV types 16, 18, 31, and 45 accounts for almost 80% of the invasive cervical cancers (pg. 19, col. 2, par. 2). Stoler further indicates that "active transcription of HPV DNA within lesions establishes a strong molecular association of HPV with cervical neoplasia" (pg. 20, col. 1, par. 2). In addition, this publication describes using HPV DNA and HPV mRNA expressed in lesions to identify patterns of viral expression, where "the presence of viral RNA and protein expression leads to a rational framework implicating the virus in lesion pathogenesis. Patterns of viral mRNA expression vary with morphology in a tightly regulated and differentiation-dependent manner" (pg. 20, 1st col., par. 1) where low-grade lesions do not have a restricted pattern of viral gene expression as found in invasive cancer. These reviews associate HPV types to cervical neoplasia, HPV genes to transformation and disease, and correlate HPV nucleic acid expression to lesion pathogenesis.

25. Saewha Jeon and Paul F. Lambert (*PNAS* 92:1654-1658, 1995; **Exhibit 14**) demonstrate that integration of HPV 16 DNA leads to increased steady-state levels of mRNAs encoding the viral oncogenes E6 and E7. Jeon and Lambert also report that expression of E6 and E7 genes in transgenic mouse systems leads to tumor formation (pg. 1654, col. 1, par. 1). This publication demonstrates the correlation of HPV gene expression, *i.e.*, E6 and E7 mRNA measurements, and disease, such as cervical cancer.

26. Goodwin and DiMaio (*PNAS* 97(23):12513-12518, 2000; **Exhibit 15**) describe that HPV 18 transcript ratios are associated with transformation, cancer, and disease stage. In particular, Goodwin and DiMaio establish that HPV 18 is associated with cervical cancer. To demonstrate the association between HPV 18 transcript measurements with transformation and disease, the authors use the HeLa cell line as a model for HPV 18-induced cervical carcinoma. Goodwin and DiMaio describe repression of HPV 18 E6 and E7 expression by inducing E2 expression, resulting in increased p53 and pRB, leading to tumor

suppression. Goodwin and DiMaio correlate E6/E7 expression and the tumor suppressor pathways, p53 and Rb, in HeLa cells (page 12517 "Implications"). The abstract states that "most cervical carcinomas express high-risk human papillomaviruses (HPVs) E6 and E7 proteins, which neutralize cellular tumor suppressor function." Since Goodwin and DiMaio report that repression of HPV oncogenes in HeLa cervical carcinoma cells induces tumor suppression, one skilled in the art understands from reading the instant application and this reference that HPV transcript measurements are associated with transformation, cancer, and disease stage.

27. Thus, it is my experience and my opinion, as one skilled in the art of HPV-induced disease, diagnosis, and treatment, that cell lines such as, but not limited to, HaCaT, W12, and SiHa are universally recognized by researchers as model systems of human epithelium at various stages of progression to cancer. It is also my opinion that the experimental results disclosed in the above-identified specification correlate to HPV-induced neoplasia, disease and cancer in humans and correlate to the diagnosis of risk, onset and stage of HPV-induced neoplasia, disease and cancer in humans. Furthermore, it is my opinion that one skilled in the art understands how to make and use the instant invention from the prior art and the instant specification.

28. It is also my opinion, as an expert in the field of nucleic acid chemistry analysis and inventor of several patents related to the use of HPV types in diagnostic testing (U.S. Patent Nos. 4,849,331, 4,849,332, 4,849,334, 4,908,306 and, 6,355,424), that the methods in the above-identified application are enabled as described in the instant specification and as commonly understood in the art.

29. To support this position, I present evidence herein establishing that the method described in the instant specification in relation to HPV 16, also applies to other high risk HPV types, such as HPV 18 and HPV 31. The data provide expression model systems for HPV 18 and HPV 31 using the E6-E7/L1 mRNA ratio assessment. The skilled artisan understands from reading the instant specification how to assess risk, onset, and stage of the HPV-induced disease in humans using other HPV genes, such as E4, E5, L2, etc.

30. The following experimental study describes an HPV 18 mRNA expression model for assessing the E6-E7/ L1 mRNA ratio. HPV 18 single-stranded DNA probes specific for E6-E7 and L1 regions were designed and developed using established protocols. HPV 18 E6-E7 and L1 RNA, complementary to the DNA probes, were transcribed *in vitro*

and used as the calibrator for normalization. The expression analysis system (EAS) protocol was utilized to obtain the expression data. The HPV 18 E6-E7/L1 mRNA ratio was measured in the HPV 18 positive human cervical carcinoma cell line, HeLa (ATCC, Manassas, VA).

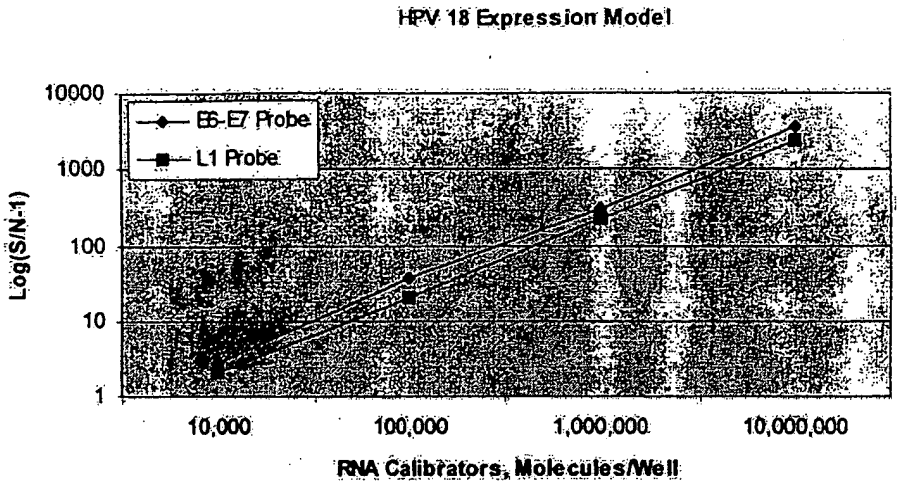
31. DNA probes were developed using PCR amplification and λ Exonuclease digestion. The following PCR primer pairs were used, where the HPV 18 E6-E7 and L1 probe sequences correspond to nucleotide regions 148-843 and 5748-6720 of the HPV 18 genome, respectively.

E6-E7 region	L1 region
5'-CTGATCTGTGCACGGAACTG-3'	5'-GGTAATCCATATTTTAGGGT-3'
5'-GCTCGAAGGTCGTCTGCT-3'	5'-CCTCAACATGTCTGCTATAC-3'

32. *In vitro* transcribed HPV 18 E6-E7 and Ll RNA were quantified and used as the calibrator for the model system. HPV 18 RNA calibrators were used at 10^3, 10^4, 10^5, 10^6, and 10^7 molecules per well.

33. The assay uses normal human keratinocytes (BioWhittaker, San Diego, CA) and HeLa cells, where 10,000 cells per well were lysed with Proteinase K (50 units per well) for 30 min at 37°C. The mixture of HPV 18 RNA calibrators, cell lysates, and DNA probe (E6-E7 or L1 at 180 pM) in hybridization buffer (Digene) was hybridized for 2 hours at 65°C. RNA/DNA hybrids were captured onto streptavidin-coated plates (Digene) for 1 hour at room temperature with agitation at 1100 rpm. Captured RNA/DNA hybrids were recognized by anti-RNA/DNA hybrid antibodies (DR-1, Digene). Incubation with DR-1 was performed for 30 min at room temperature. The plate was washed with HCII Wash buffer (Digene) 4 times, incubated with Enhance buffer (Digene) for 45 min at 53°C, and washed again 4 times with HCII Wash buffer. The plate was incubated with DR-2 (Digene) for 15 min at room temperature, and then read on a luminometer. HPV 18 negative normal human keratinocytes were used as a background signal (N; Noise) to assess the HPV E6-E7 and L1 mRNA levels measured from HPV 18 positive HeLa cells. Both cell lines were used at the same concentrations, 10,000 cells/well.

GRAPH 1



RNA Calibrators, Molecules/Well	10,000	100,000	1,000,000	10,000,000
E6-E7 Probe, S/N-1	2.7	37.3	306.4	3549.7
L1 Probe, S/N-1	2.1	20.6	219.8	2404.3

Table 1. HPV 18 Expression Model: The analytical sensitivity of the HPV 18 probes is depicted in Graph 1 and Table 1 ($intercept_{E6\text{-}E7}$= -5.14, $slope_{E6\ E7}$=1.28; intercepts=-3.85, $slopes_{L1}$=1.03).

Cells, 10,000 per well	Average Negative RLU (Keratinocytes)	Average Positive RLU (HeLa)	%CV	S/N	Total mRNA	mRNA/cell
E6-E7	79	53,988	6.1	683.4	1,749,217	175
L1	153	6,142	26.5	40.1	184,963	18

Table 2. HPV 18, E6-E7 and L1 mRNA in HeLa: HPV 18 E6-E7 and L1 mRNA detection using the HeLa cell line. The assay was run in triplicate.

Cell Line	E6-E7/L1 Ratio
HeLa	9.5

Table 3. The HPV 18 E6-E7/L1 mRNA Ratio.

34. The E6-E7/L1 mRNA ratio was found to be 9.5 for HPV 18 positive human cervical carcinoma cell line HeLa. This E6-E7/L1 mRNA ratio correlates to the claimed invention, i.e. This ratio represents substantially higher expression of the carcinogenesis-related genes E6-E7 than the viral capsid structural L1 gene.

35. As further evidence that the claimed HPV gene transcript ratio applies to other HPV types, an HPV 31 mRNA expression model was developed for the E6-E7/L1 mRNA ratio assessment. HPV 31 single-stranded DNA probes specific for E6-E7 and L1 regions were designed and developed using established protocols. Full-length HPV 31 RNA, complementary to the DNA probes, was transcribed *in vitro* and used as the calibrator for normalization. The EAS protocol was utilized to obtain the expression data. The HPV 31 E6-E7/L1 mRNA ratio was measured in total cellular RNA isolated from the HPV 31 positive neoplastically transformed cell lines LKP31 and A31 (a gift from Dr. L.A. Laimins, Northwestern University).

36. DNA probes were developed using PCR amplification and λ Exonuclease digestion. The following PCR primers pairs were used, where the HPV 31 E6-E7 and L1 probe sequences correspond to nucleotide regions 96-803 and 5964-7017 of the HPV 31 genome, respectively.

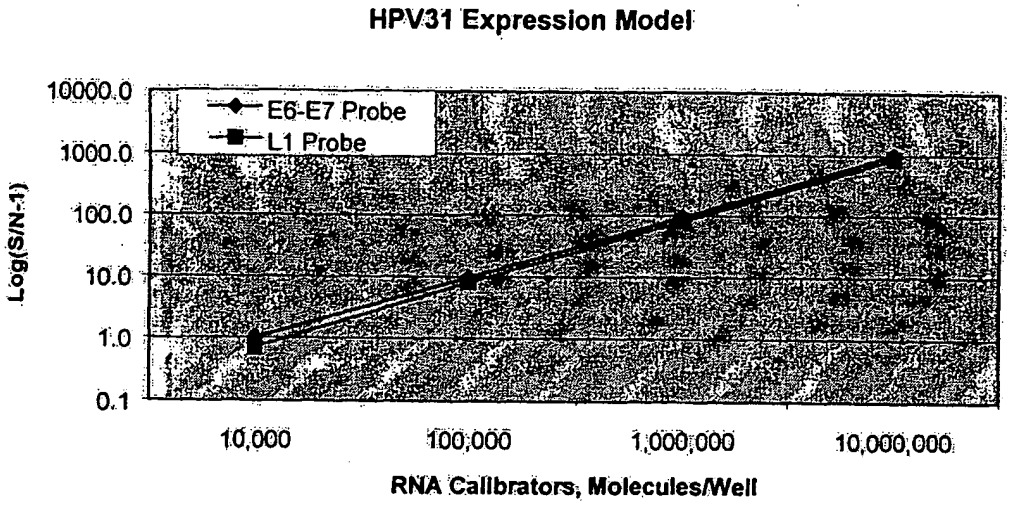
E6-E7 region	L1 region
5'-CCTACAGACGCCATGT-3'	5'-GTGGTCCTGGCACTGATAAT-3'
5'-GCTCTTGCAATATGCGAATA-3'	5'-GGGTGCACTACGTTTACCTG-3'

37. *In vitro* transcribed full-length HPV 31 RNA was quantified and used as the calibrator for the model. HPV 31 RNA calibrators were used at 10^4, 10^5, 10^6, and 10^7 molecules per well.

38. The assay uses a mixture of HPV 31 RNA from the calibrators or isolated from cell lines and DNA probe (E6-E7 at 360 pM and L1 at 1500 pM) in hybridization buffer (Digene). Hybridization occurred for 2 hours at 65°C. RNA/DNA hybrids were captured onto streptavidin-coated plates (Digene) for 1 hour at room temperature with agitation at 1100 rpm. Captured RNA/DNA hybrids were recognized by anti-RNA/DNA hybrid antibodies (DR-1, Digene). Incubation with DR-1 was performed for 30 min at room temperature. The plate was washed with HCII Wash buffer (Digene) 4 times, incubated with Enhance buffer (Digene) for 45 min at 53°C, and washed again 4 times with HCII Wash buffer. The plate was incubated with DR-2 (Digene) for 15 min at room temperature, and then read on a luminometer. The total cellular RNA samples, isolated from human keratinocytes were transfected with HPV 31 DNA. Both cell lines utilized in this assay, LKP31 and A31, contained episomal and integrated copies of HPV 31 DNA; however, LKP31 had a higher copy number than A31, and thus LKP31 is assumed to represent a cell

line that is closer to cancer. E6-E7 and L1 mRNA expression analysis was performed using 500 ng per well of the cellular RNA sample. The 500 ng of total RNA approximately corresponded to $1.4x10^4$ cells (based on Qiagen RNeasy Handbook, 35 µg of total RNA ~ 10^6 cells, and Digene experimental data, 32 µg of total RNA $2x10^6$ cells).

Graph 2



RNA Calibrators, Molecules/Well	10,000	100,000	1,000,000	10,000,000
E6-E7 Probe, S/N-1	1.0	9.6	95.2	1007.1
L1 Probe, S/N-1	0.7	8.0	81.4	861.2

Table 4. HPV 31 Expression Model: The analytical sensitivity of the HPV 31 probes is depicted in Graph 2 and Table 4 ($intercept_{E6-E7}$= -4.05, $slope_{E6-E7}$=1.01; $intercepts_{L1}$=-4.23, $slopes_{L1}$=1.02).

Total RNA 500 ng/well	Average Negative RLU	Average Positive RLU	%CV	S/N	Total mRNA	mRNA/cell
LKP31	36.3	25,836	25.7	711.1	7,186,810	513
A31	36.3	11,174	1.0	307.5	3,120,083	223

Table 5. HPV 31 E6-E7 mRNA. Specimens were tested in duplicate.

Total RNA 500 ng/well	Average Negative RLU	Average Positive RLU	%CV	S/N	Total mRNA	mRNA/cel l
LKP31	51.7	2,615	14.8	50.6	612,792	44
A31	51.7	1,584	0.3	30.7	370,737	27

Table 6. HPV 31 L1 mRNA detection using the LKP31 and A31 total RNA samples. Specimens were tested in duplicate.

Cell Line	E6-E7/L1 Ratio
LKP31	11.7
A31	8.4

Table 7. The HPV 31 E6-E7/L1 mRNA Ratio.

39. The E6-E7/L1 mRNA ratio was found to be above 2 for both HPV 31 positive cell lines LKP31 and A31. This ratio represents higher expression of the carcinogenesis-related genes E6-E7 than the viral capsid structural L1 gene. The levels of E6-E7 and L1 mRNA were approximately 2-fold higher in LKP31 cells than in the A31 cells. The higher level of E6-E7 to L1 is expected in cells that are transformed by HPV 31 to the pre-malignant state, and as may be expected, the LKP31 cell line had a higher E6-E7 to L1 ratio, possibly indicating a more neoplastic cell line.

40. These experiments demonstrate that HPV gene transcript ratios of different HPV types, *i.e.*, HPV 18 and HPV 31, may be used to determine the disease level in cell model systems of HPV-infected cells. In particular, the expression levels of E6-E7 and L1 mRNA were detected; however, one skilled in the art may, from reading the instant specification, determine the HPV gene transcript ratio of other HPV genes than E6-E7 and L1.

41. In addition to the data previously presented, Mark Stoler ("Human Papillomaviruses and Cervical Neoplasia: A Model for Carcinogenesis" *Int. J. Gynecol. Pathol.*, 19:16-28, 2000; **Exhibit 16**), describes the varying risk types of HPV at page 18, left column, which include high risk HPV 16, 18, 31, 33. Stoler further establishes that active transcription of HPV DNA is associated with cervical neoplasia and the expression of the E6 and/or E7 region (page 20, left col., first full paragraph).

42. Louise Chow and Thomas Broker (Chapter 12, pages 279-280 of "Small DNA Tumor Viruses" *Viral Pathogenesis*, edited by Neal Nathanson, et al. Lippincott-Raven Publishers, Philadelphia © 1997) report of high risk HPVs and the genes associated with them. In particular, E6, E7 gene expression is increased in high-grade lesions and carcinomas, while E1, E2, E4, and E5 is low. L1 and L2 signals are found to be absent from high-grade lesions. The expression of these genes correlate to high risk HPV types, including but not limited to HPV16, HPV18, HPV31, and HPV33. Accordingly, one skilled in the art would understand the correlation between the HPV types and HPV-induced neoplasia and how to determine the risk of HPV-induced neoplasia from the claimed HPV mRNA ratios.

43. The cell lines such as HeLa, W12, SiHa are widely recognized by scientists as model systems of human epithelium in a patient at various stages progressing to cancer, where these cell lines contain different HPV copy numbers per cell. It is my opinion that the instant specification and prior art at the time of filing enables one skilled in the art to diagnose and prognose a patient having an HPV-induced disease using HPV gene transcripts as an indicator. In addition, the specification teaches and provides guidance for the skilled artisan to measure and analyze HPV gene transcript ratios for diagnosis of disease caused by different HPV types.

44. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Respectfully submitted,

Date: 9/30/04

Attila T. Lorincz, Ph.D.

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HUMAN PAPILLOMAVIRUS IS A NECESSARY CAUSE OF INVASIVE CERVICAL CANCER WORLDWIDE

JAN M. M. WALBOOMERS[1]*, MARCEL V. JACOBS[1], M. MICHELE MANOS[2], F. XAVIER BOSCH[3], J. ALAIN KUMMER[1], KEERTI V. SHAH[2], PETER J. F. SNIJDERS[1], JULIAN PETO[4], CHRIS J. L. M. MEIJER[1] AND NUBIA MUÑOZ[5]

[1]*Department of Pathology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands*
[2]*Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore, MD, U.S.A.*
[3]*Institute Catala d'Oncologia, Barcelona, Spain*
[4]*Institute of Cancer Research, Belmont, Surrey, U.K.*
[5]*Unit of Field and Intervention Studies, IARC, Lyon, France*

SUMMARY

A recent report that 93 per cent of invasive cervical cancers worldwide contain human papillomavirus (HPV) may be an underestimate, due to sample inadequacy or integration events affecting the HPV L1 gene, which is the target of the polymerase chain reaction (PCR)-based test which was used. The formerly HPV-negative cases from this study have therefore been reanalysed for HPV serum antibodies and HPV DNA. Serology for HPV 16 VLPs, E6, and E7 antibodies was performed on 49 of the 66 cases which were HPV-negative and a sample of 48 of the 866 cases which were HPV-positive in the original study. Moreover, 55 of the 66 formerly HPV-negative biopsies were also reanalysed by a sandwich procedure in which the outer sections in a series of sections are used for histological review, while the inner sections are assayed by three different HPV PCR assays targeting different open reading frames (ORFs). No significant difference was found in serology for HPV 16 proteins between the cases that were originally HPV PCR-negative and -positive. Type-specific E7 PCR for 14 high-risk HPV types detected HPV DNA in 38 (69 per cent) of the 55 originally HPV-negative and amplifiable specimens. The HPV types detected were 16, 18, 31, 33, 39, 45, 52, and 58. Two (4 per cent) additional cases were only HPV DNA-positive by E1 and/or L1 consensus PCR. Histological analysis of the 55 specimens revealed that 21 were qualitatively inadequate. Only two of the 34 adequate samples were HPV-negative on all PCR tests, as against 13 of the 21 that were inadequate ($p<0{\cdot}001$). Combining the data from this and the previous study and excluding inadequate specimens, the worldwide HPV prevalence in cervical carcinomas is 99·7 per cent. The presence of HPV in virtually all cervical cancers implies the highest worldwide attributable fraction so far reported for a specific cause of any major human cancer. The extreme rarity of HPV-negative cancers reinforces the rationale for HPV testing in addition to, or even instead of, cervical cytology in routine cervical screening.

KEY WORDS—cervical carcinoma; HPV PCR; histology; HPV serology; epidemiology

INTRODUCTION

Cervical cancer is the second commonest cancer in women worldwide and infection with oncogenic human papillomavirus (HPV) types, most frequently HPV 16, is the most significant risk factor in its aetiology.[1] The International Biological Study on Cervical Cancer (IBSCC) study of invasive cervical cancers collected from 22 countries reported a worldwide HPV prevalence of 93 per cent, based on the MY09/11 polymerase chain reaction (PCR) assay which targets a 450 base pair (bp) fragment within the HPV L1 open reading frame (ORF).[2] Failure to detect HPV DNA in 7 per cent of these cervical carcinomas may have been due to either the absence of HPV DNA in the carcinoma cells, or a false-negative HPV result. Integration of HPV DNA in cervical carcinoma can entail disruption of PCR primer sequences or loss of the HPV L1 ORF.[3] In contrast, the E6 and E7 genes are almost invariably retained, as their expression is likely to be necessary for conversion to and maintenance of the malignant state.[4–8] A false negative can also be due to the absence of cancer cells from the sample analysed.

This study was undertaken to reassess the prevalence of HPV DNA in this worldwide series of cervical cancers. Since a marked association has been reported between cervical cancer and antibody responses against HPV-specific proteins,[9–13] serological analysis was first performed for capsid and E6 and E7 proteins of HPV 16, which accounts for about 50 per cent of HPV-positive cervical cancers worldwide.[2] To verify the presence of carcinoma cells in the samples used for PCR, formalin-fixed, paraffin-embedded biopsies of the HPV-negative cases were cut using a sandwich technique, in which the inner sections were used for PCR while the outer sections were used for histological review. The efficacy of amplification in paraffin-embedded tissue specimens is inversely related to the length of the fragment to be amplified.[14] The analysis was therefore restricted to samples which amplified at least 200 bp, as

*Correspondence to: Professor Dr. J. M. M. Walboomers, Department of Pathology, University Hospital Vrije Universiteit, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands.
E-mail: JMM.Walboomers@azvu.nl

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determined by β-globin PCR. Adequate samples were assayed with 14 type-specific (TS) PCRs for high-risk HPV types targeting approximately 100 bp in the E7 ORF. In addition, CPI/II and GP5+/6+ consensus primer-mediated PCR assays were used, which also target small fragments. The PCR results were correlated with the histological findings.

MATERIALS AND METHODS

Clinical specimens

Sera from HPV-negative and a tissue sample from the HPV-positive cervical cancer patients were obtained from the IBSCC Study.[2] The HPV-positive cases were matched in frequency to the negative cases on age, histological type, clinical stage, and area of residence.

In the original analysis, 866 snap-frozen biopsies from patients with cervical cancer were HPV-positive and 66 were HPV-negative by MY09/11 PCR.[2] For the present study, formalin-fixed, paraffin-embedded tissue specimens were obtained from 58 of the 66 HPV-negative cases. Material from the remaining eight negative cases was not available.

A series of 5 μm sections were cut; the outer sections were used for histological analysis while the inner sections were used for PCR.[15] Depending on the size of the biopsy, five to ten sections were cut. For PCR analysis, tissue sections were pretreated as described previously.[16]

HPV serology

Antibodies to HPV 16 virus-like particles (VLPs) were detected by ELISA[17] and antibodies to HPV 16 E6 and E7 proteins were measured by a radioimmunoprecipitation assay (RIPA) with *in vitro* translated ^{35}S-labelled full-length E6 and E7 proteins.[11]

β-Globin PCR

β-Globin PCRs were performed[18] using four primer combinations spanning 100, 209, 326, and 509 bp to assess the quality of the DNA.

HPV E7 type-specific PCRs

For E7 PCRs, HPV type-specific oligonucleotides were selected on the basis of sequence information from the HPV sequence database[19] after alignment analysis using the Clustal program (PC/Gene, Release 6.7; Intelligenetics, Inc, Geneva Switzerland). HPV type-specific primers were chosen to amplify approximately 100 bp in the E7 ORF of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Type-specific (TS) PCRs were performed under the conditions described by van den Brule *et al.*[20] HPV genotypes were identified by Southern blot hybridization using type-specific oligonucleotide probes. Reconstruction experiments using cloned HPV DNA serially diluted in human placental DNA revealed a sensitivity of between 10 and 100 HPV copies per sample for all primer sets (data not shown). Oligonucleotide sequences used as primers and probes are shown in Table I.

Consensus primer-mediated PCRs

Consensus PCR primers GP5+/6+[21] and CPI/II[22] were used for amplification. GP5+/6+ PCR was performed with one biotinylated primer (bioGP6+) to enable subsequent typing in an enzyme immunoassay.[23] CPI/II PCR was performed as described elsewhere.[22] Both consensus PCRs amplify a broad spectrum of HPV types at the subpicogram level. HPV positivity was assessed by Southern blot analysis of the PCR products with general probes of HPV-specific [α-^{32}P]dCTP-labelled DNA fragments derived from cloned DNA of HPV 6, 11, 16, 18, 31, and 33 as described previously.[21]

PCR products from GP5+/6+ PCR-positive specimens were typed using internal HPV type-specific oligonucleotide probes for the most common HPV types 16, 18, 31, and 33. The PCR products of CPI/II PCR-positive specimens were typed by stringent Southern blot analysis with [α-^{32}P]dCTP random-primed labelled full-length cloned HPV DNA of types 16, 18, 31, and 33 as described elsewhere.[24]

Negative controls for PCR analysis

Several precautions were taken to prevent false-positive results. Different steps such as sample preparation and amplification were performed in strictly separated rooms, and distilled water samples were included as negative PCR controls. To monitor sample-to-sample carry-over, HPV-negative liver tissue was cut after each specimen and subjected to all subsequent procedures including HPV PCR. None of these control samples was positive for any of the HPV assays.

Histological review

The first and last sections of each tissue were haematoxylin and eosin (H&E)-stained for histological analysis. The slides were examined by a pathologist without knowledge of the HPV status for the presence of neoplastic cells, extent of necrosis, extent of keratinization, and evidence of maltreatment (i.e. nuclear vacuolation suggestive of freeze/thaw damage). Final classification of equivocal specimens was made jointly with a second pathologist.

Statistical analysis

The Mann–Whitney *U*-test was used to compare OD and cpm values between HPV-positive and -negative cases. Histopathological characteristics were compared using Fisher's exact test. All significance levels are two-sided.

RESULTS

The 66 HPV-negative and 866 HPV-positive cases in the IBSCC study were similar in relation to established

Table I—Oligonucleotide sequences of HPV E7 type-specific PCRs

Primer	Sequence (5′–3′)	Probe	Sequence (5′–3′)
HPV16E7.667	gatgaaatagatggtccagc	PROHPV16E7	cggacagagcccattacaatattgtaacct
HPV16E7.774	gctttgtacgcacaaccgaagc		
HPV18E7.696	aagaaaacgatgaaatagatgga	PROHPV18E7	cccgacgagccgaaccacaacgtcacacaa
HPV18E7.799	ggcttcacacttacaacaca		
HPV31E7.811	gggctcatttggaatcgtgtg	PROHPV31E7	tacctgctggatcagccattgtagttacag
HPV31E7.890	aaccattgcatcccgtcccc		
HPV33E7.671	tgaggatgaaggcttggacc	PROHPV33E7	tgtgacaacaggttacaatgtagtaatcag
HPV33E7.761	tgacacataaacgaactgtg		
HPV35E7.674	ctattgacggtccagct	PROHPV35E7	caacaggacgttacaatattataattggag
HPV35E7.752	tacacacagacgtagtgtcg		
HPV39E7.601	ccaaagcccaccttgcagga	PROHPV39E7	tcctaattgctcgtgacatacaaggtcaac
HPV39E7.723	atggtcgggttcatctatttc		
HPV45E7.741	cccacgagccgaaccacag	PROHPV45E7	agctcaattctgccgtcacacttacaacat
HPV45E7.822	tctaaggtcctctgccgagc		
HPV51E7.718	tacgtgttacagaattgaag	PROHPV51E7	tcaagtgtagtacaactggcagtggaaagc
HPV51E7.841	aaccaggcttagttcgcccatt		
HPV52E7.691	gcagaacaagccacaagcaa	PROHPV52E7	atagccgtagtgtgctatcacaactgtgac
HPV52E7.776	tagagtacgaaggtccgtcg		
HPV56E7.784	ggtgcagttggacattcagag	PROHPV56E7	caaagaggacctgcgtgttgtacaacagct
HPV56E7.886	gttacttgatgtcgcagagtg		
HPV58E7.98	cgaggatgaaataggcttgg	PROHPV58E7	tgttgttcaatgttacatcattaatcgaca
HPV58E7.761	acacaaacgaaccgtggtgc		
HPV59E7.646	ctccgagaatgaaaaagatgaa	PROHPV59E7	gtcgagcagatcgatcatcgtttcctacta
HPV59E7.749	gctgaagttgattattaca		
HPV66E7.641	aatgcaatgagcaattggacag	PROHPV66E7	aggatgaaatagaccatttgctggagcggc
HPV66E7.742	cttatgttgttcagcttgtc		
HPV68E7.4604	aacaacagcgtcacacaattca	PROHPV68E7	agtgtaacaacctactgcaactagtagtag
HPV68E7.4704	agttgtacgttccgcaggtt		

risk factors for cervical cancer (geographical origin, socio-economic status, sexual and reproductive lifestyle, exposure to cigarette smoke, hormonal treatments, and history of venereal disease).[2]

Serological analysis

Sera were obtained from 49 of the 66 HPV PCR-negative cases (group 1) and from a matched sample of 48 initially positive cases (group 2) from the previous study.[2] Reactivity to HPV 16 VLPs and E6 and E7 proteins was detected in 31 per cent (16/49), 35 per cent (17/49), and 29 per cent (14/49) of group 1 and in 42 per cent (20/48), 52 per cent (25/48), and 31 per cent (15/48) of group 2, respectively. Serum antibodies to at least one protein were detected in 56 and 67 per cent of groups 1 and 2, respectively. Positive serum samples tended to have lower antibody levels for VLPs and E6 in group 1 than in group 2 (Fig. 1), but there was no significant difference in percentage reactivity to VLPs ($p=0{\cdot}92$), E6 ($p=0{\cdot}30$) or E7 ($p=0{\cdot}76$) between the groups.

PCR analyses

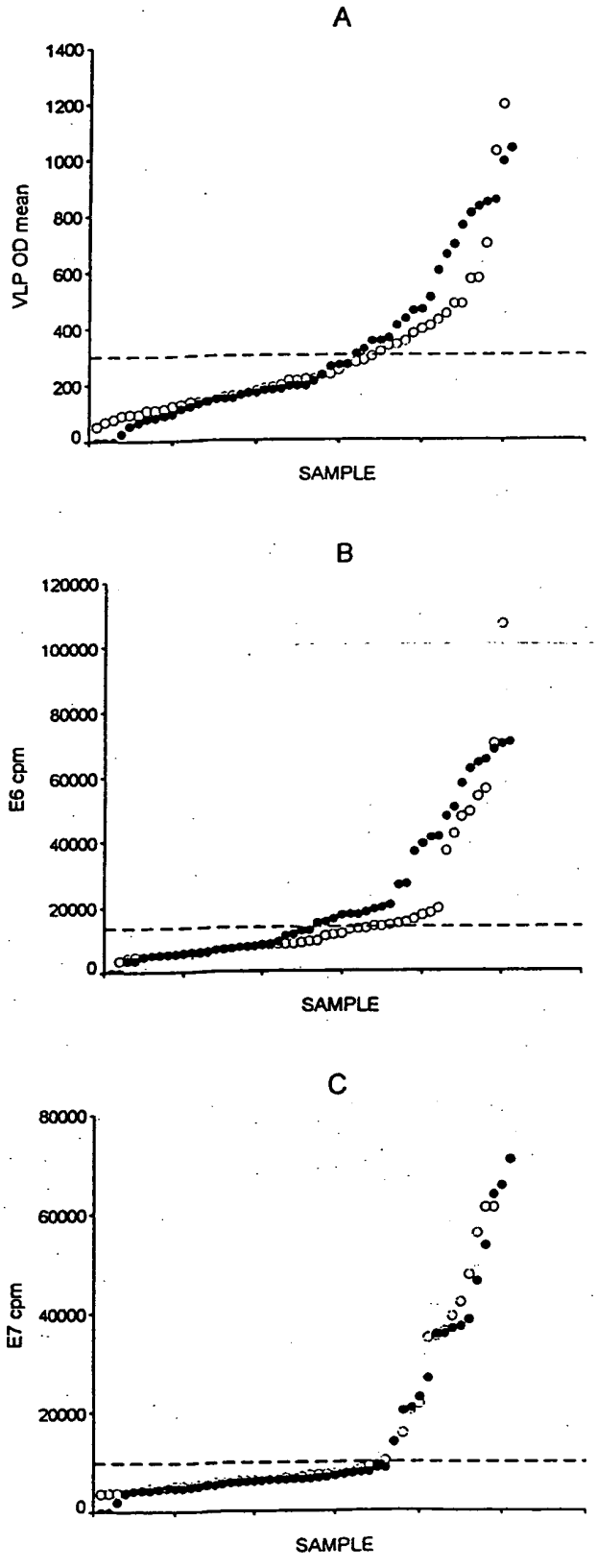
The integrity of the target DNA was determined by β-globin PCR assays amplifying different fragment lengths. Of the 58 cervical carcinoma specimens, 55 (95 per cent) were positive for amplification of β-globin fragments of 100 and 209 bp, and 43 (74 per cent) and 21 (36 per cent) cases were also positive for 326 and 509 bp fragments, respectively. The three cases which were negative with all four β-globin primer sets were excluded from the HPV PCR assays, all of which entailed amplification of sequences of 200 bp or less.

HPV E7 PCRs on the remaining 55 specimens were positive for one or more HPV types in 38 (69 per cent) cases. HPV 16 was detected in 15 cases; HPV 18 in nine cases; HPV 31 in four cases; HPV 45 in two cases; and HPV 33, 39, 52, and 58 in one case each. Multiple HPV infections were found in four cases: HPV 16/18 twice and HPV 18/45 and HPV 31/33 once each.

Using consensus PCRs, 14 samples were positive only with E1 primers, six only with L1 primers, and ten with both. Two cases were positive by consensus PCR but negative by E7 PCR. One of these was positive only for E1 and the other for both E1 and L1. These cases therefore contain an HPV type different from the 14 HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) tested for by HPV E7 PCR. Tentatively, these types were referred to as HPV-X. The remaining 28 consensus PCR-positive samples were also positive by E7 PCR, and in every case in which typing of L1 and E1 PCR products indicated HPV 16, 18, 31 or 33, the same type was detected by E7 PCR.

Using HPV E7 type-specific and consensus L1 and E1 PCRs, the number of HPV-positive cases was thus 40/55

(73 per cent), while 15/55 (27 per cent) cases remained HPV-negative. The HPV detection and typing results are summarized in Table II.



Histological review of cervical carcinoma biopsies

The histological results and HPV status of the 55 biopsies are shown in Table III. Among the 40 HPV-positive cases, 31 were diagnosed as squamous cell carcinomas (SCC) and one as adenosquamous carcinoma. Six showed only dysplastic epithelium and the remaining two specimens were inadequate, one due to extensive necrosis (>75 per cent) and one due to maltreatment. Of the 32 HPV-positive histologically adequate cases, 24 (75 per cent) were positive on at least two different PCR assays. Two of the 15 HPV-negative cases were SCCs. The remaining 13 cases were characterized as having only dysplastic ($n=1$) or normal ($n=2$) epithelium, only stroma ($n=2$), extensive necrosis (>75 per cent; $n=2$), maltreatment ($n=3$) or extensive keratinization (>75 per cent; $n=3$). The histology of the HPV-negative group is shown in Fig. 2. The material was thus inadequate for eight (20 per cent) specimens in the HPV-positive group compared with 13 cases (87 per cent) in the HPV-negative group ($p<0{\cdot}001$). Figure 3 summarizes the PCR results in relation to adequacy of the tissue.

DISCUSSION

This study was designed to investigate the extent of true HPV-negative cervical cancer by retesting the cases originally classified as HPV-negative in a worldwide study of HPV prevalence in cervical carcinomas.[2] Samples from 58 of the 66 HPV-negative cases were available, and 55 were adequate for PCR analysis. The similarity of the antibody profiles of HPV-negative and -positive cases (Fig. 1) suggests that a significant proportion of the HPV-negative group were in fact HPV 16-associated. The epidemiological characteristics of the positive and negative cases were also similar. Our histological reassessment and PCR assays targeting different ORFs used cervical cancer samples prepared by the sandwich method, which addresses two potentially important causes of false-negative results: disruption of PCR target sequence due to viral integration, and inadequacy of the specimens.

The results of our consensus PCR assays targeting small fragments in the L1 (GP5+/6+ PCR) and E1 (CPI/II PCR) regions, which were positive in only about 50 per cent of the E7 PCR-positive cases (Fig. 3), suggest the presence of interruptions or deletions in the HPV DNA at the level of the L1 ORF. Our PCR assays achieve similar sensitivity for each HPV type, so these findings indicate that in the remaining E7-positive cases disruptions were present in both L1 and E1. Although integration assays are necessary ultimately to

Fig. 1—Antibody responses to HPV 16 VLP (A), E6 (B), and E7 (C) proteins of formerly HPV-negative (○) and HPV-positive (●) cases of cervical cancer. For VLP reactivity, mean extinctions (mean OD) and for E6/E7 reactivity, the counts per minute (cpm) are indicated. The cut-off levels for seropositivity (mean+3 × SD) for VLP (300), E6 (13 556), and E7 (9714) are indicated by dotted lines. Sera are arrayed in order of increasing reactivity. Each dot represents one serum specimen

Table II—Overall HPV prevalence and type distribution*

			HPV genotyping											
n	HPV−	HPV+	16	18	31	33	39	45	52	58	16/18	18/45	31/33	X†
55	15	40	15	9	4	1	1	2	1	1	2	1	1	2

*HPV prevalence was determined by HPV E7 type-specific PCR and consensus GP5+/6+ and CPI/II PCR assays. HPV genotyping was performed by HPV E7 type-specific PCRs.
†HPV-X: HPV types different from HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 as detected by GP5+/6+ and CPI/II PCR.

substantiate that integration is one of the underlying causes of false negativity, the suggestion that the disruptions are due to integration events was supported by further typing analysis. The prevalence of HPV 18 in the original analysis was more than three-fold lower than that of HPV 16 (13·7 per cent versus 49·9 per cent),[2] but amongst the E7-positive cases, HPV 18 was almost as prevalent as HPV 16 (22 per cent versus 31 per cent), suggesting that HPV 18 is more often disrupted in the L1 and/or E1 region. Previous studies[25,26] have shown that almost all HPV 18-positive cervical carcinomas contain only integrated HPV 18 DNA. In contrast, the proportion of HPV 16-positive cases that contain only integrated viral genomes is 60 per cent or less.[25–27] In a previous study, E7 ORF transcripts were detected in the neoplastic cells of all HPV 16 DNA-positive cervical carcinomas, including cases with very low HPV 16 copy number, provided that the RNA quality of the tissue was adequate.[15] This demonstration of continuous expression of the E7 ORF indicates an active role of E7 in the pathogenesis of cervical cancer. The samples in the present study were inadequate for RNA *in situ* hybridization (RISH), but the presence of E7 DNA in all 37 specimens in which DNA from a known HPV type was detected, including 23 without detectable L1 DNA and 16 without detectable E1 DNA (Fig. 3), supports the conclusion that E7 is required for maintenance as well as for initiation of the malignant state.[8]

Our PCR results thus indicate that many of the samples which were HPV-positive in this study were false-negative in the previous study due to integration events affecting MY09/11 L1 sequences, as suggested by Bosch *et al.*[2] It is most unlikely that the additional positives scored in this study are due to contamination. None of the negative controls included during sample preparation and PCR testing gave a positive result, and as the great majority were positive on two or more PCR assays, the results cannot be due to contamination with specific PCR products (Fig. 3). The strongest evidence, however, is our striking observation that a large majority (87 per cent) of HPV-negative cases, but only a minority (20 per cent) of HPV-positive cases (Table III: $p<0·001$), were histologically inadequate, which cannot be an artefact of contamination. This suggests that failure to detect HPV DNA in these cases in the original study, in which the presence of carcinoma was diagnosed in adjacent blocks, was due mainly to inadequate sampling. Histological inadequacy was defined in the present study by the presence of only normal or dysplastic epithelium or stroma, extensive necrosis, maltreatment, or extensive keratinization (Fig. 2). When analysis was restricted to samples which were histologically adequate, HPV was detected in 94 per cent (32/34) of the formerly HPV-negative carcinomas. The two remaining HPV-negative samples, which contained 50–75 per cent cancer cells and occasional necrosis, may be true HPV negatives, or might contain viral variants, sequence disruptions or very low HPV levels that escaped detection by the methods used.

Table III—Correlation of HPV status and histological characteristics

Histology	HPV+ (n=40) *N* (%)	HPV− (n=15) *N* (%)
Squamous cell carcinoma	31 (77·5)	2 (13·3)
Adenosquamous carcinoma	1 (2·5)	0 (0)
Only dysplastic or normal epithelium present	6 (15)*	3 (20)†
Extensive necrosis	1 (2·5)	2 (13·3)
Maltreatment	1 (2·5)	3 (20)
Only stroma present	0 (0)	2 (13·3)
Extensive keratinization	0 (0)	3 (20)

*All samples showed dysplastic epithelium.
†Two samples showed normal epithelium; one was dysplastic.

The great majority of cases in the IBSCC study were squamous carcinomas, but the conclusion that almost all cervical cancers contain HPV DNA is likely to be true irrespective of histology. Among cases which were adenocarcinomas or adenosquamous carcinomas, the prevalence of HPV by L1 consensus PCR was 95 per cent, the majority being HPV 18 or related types,[2] and the single adequate sample which was initially HPV-negative was shown in the present study to contain HPV E7 DNA (Table III). The IBSCC study included only 43 adenocarcinomas or adenosquamous carcinomas, but recent studies[28–30] using similar consensus PCR methods also detected HPV L1 DNA in 90 per cent or more of cervical cancers of these histologies, with HPV 18 being the most prevalent type. Our results suggest that HPV 18 is more often disrupted in the L1 region than other HPV types, which would imply that PCR assays targeting other ORFs are required to determine the true prevalence in adenocarcinomas.

The results of the IBSCC report together with the present study indicate that virtually all cervical cancers contain HPV DNA. In the original analysis, 981 samples were analysed by consensus PCR; 115 of these were

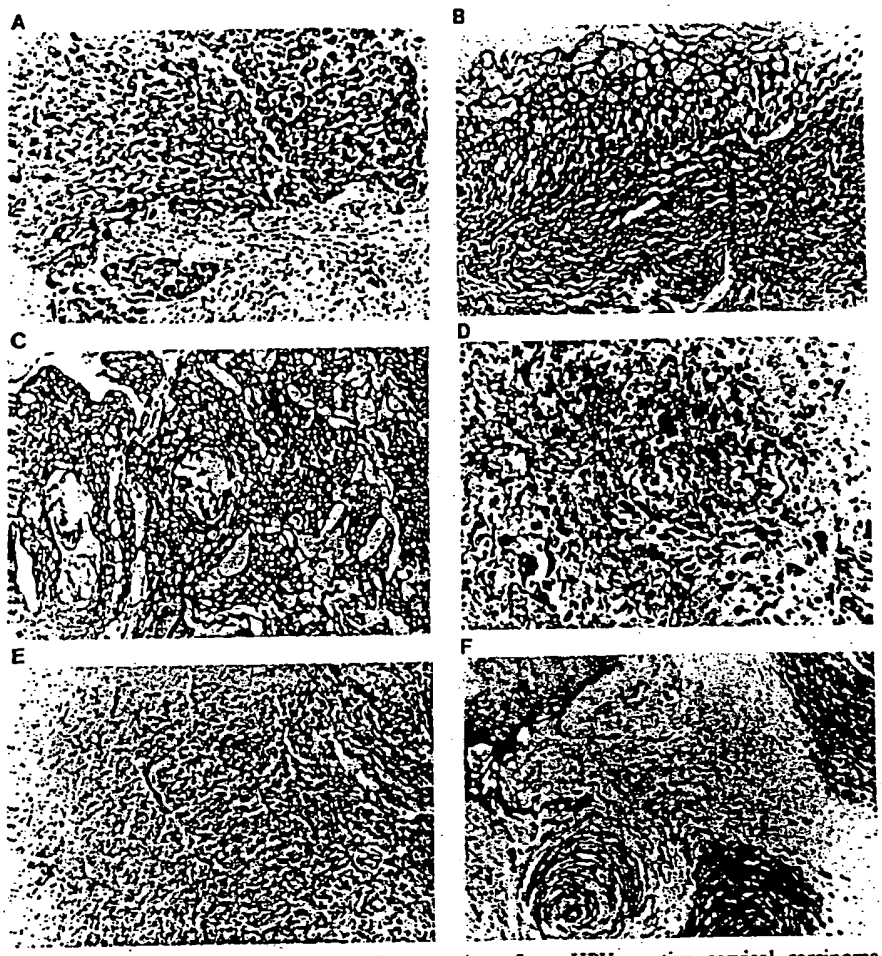


Fig. 2—Compilation of H&E-stained tissue sections from HPV-negative cervical carcinoma biopsies. (A) Squamous cell carcinoma; (B) only dysplastic/normal epithelium; (C) serious maltreatment, i.e. nuclear vacuolation; (D) extensive necrosis (>75 per cent); (E) only stroma; (F) extensive keratinization (>75 per cent)

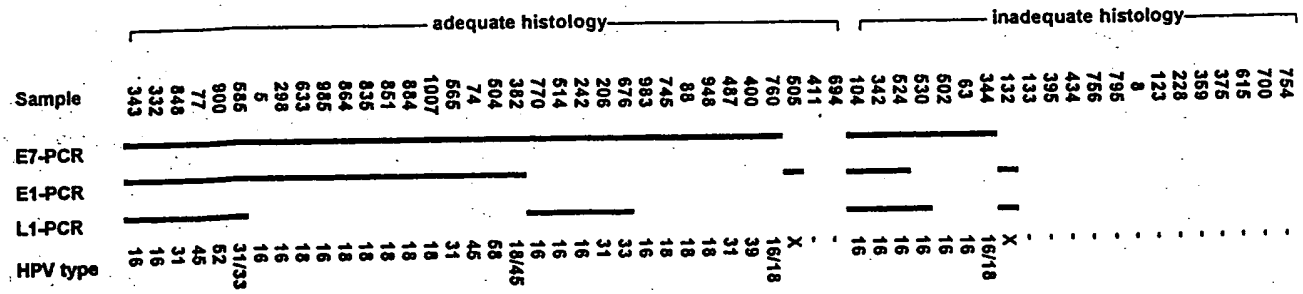


Fig. 3—HPV analysis of 55 cervical carcinomas using different PCR assays in relation to tissue adequacy. Bars indicate positivity for the respective PCR assay. HPV-X is HPV E7 type-specific PCR-negative but positive with one or both consensus PCR assays. E1 PCR: CP1/II PCR; L1 PCR: GP5+/6+ PCR

HPV-negative, of which 66 (57 per cent) were found on review to be adequate (containing tumour and PCR-sufficient).[2] Of these 66 samples, 58 were examined in the present study, and under our more stringent review (recutting multiple sections and accepting only cases with tumour in the outer sections and PCR-detectable β-globin DNA in the inner sections), 34 (59 per cent) were adequate, implying that only 34 per cent ($0{\cdot}57 \times 0{\cdot}59$) of the 115 originally negative specimens were adequate by this series of procedures. The proportion of the 866 initially HPV-positive specimens that would have been adequate by the same criteria is unlikely to be less than 80 per cent, the proportion that we observed among the 40 initially negative specimens which were found to be HPV-positive on retesting in the present study. On this assumption, the original study

included about 693 (80 per cent of 866) positive and 39 (34 per cent of 115) initially negative specimens which were adequate by our criteria. Our finding that only 6 per cent (2/34) of initially negative adequate specimens remained HPV-negative on retesting thus implies an overall HPV prevalence of 99·7 per cent among cervical cancers worldwide. Even if half of all women in the sampled populations had been infected with HPV at some time in their lives, this would indicate the highest worldwide attributable fraction ever identified for a specific cause of a major human cancer. The virtual absence of HPV-negative cancers implies that effective prophylactic vaccination might almost eliminate cervical cancer worldwide.[31] This is especially relevant in less developed countries, where screening may not be economically feasible. For developed countries, our results reinforce the rationale for HPV testing in combination with, or even instead of, cytology in population-based screening programmes.[32]

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Papillomavirus infections – a major cause of human cancers

Harald zur Hausen *

Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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Contents

* Corresponding author. Fax: +49 6221 422840.

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1. Summary

The papillomavirus family represents a remarkably heterogeneous group of viruses. At present, 77 distinct genotypes have been identified in humans and partial sequences have been obtained from more than 30 putative novel genotypes. Geographic differences in base composition of individual genotypes are generally small and suggest a low mutation rate and thus an ancient origin of today's prototypes. The relatively small size of the genome permitted an analysis of individual gene functions and of interactions of viral proteins with host cell components. Proliferating cells contain the viral genome in a latent form, large scale viral DNA replication, as well as translation and functional activity of late viral proteins, and viral particle assembly are restricted to differentiating layers of skin and mucosa.

In humans papillomavirus infections cause a variety of benign proliferations: warts, epithelial cysts, intraepithelial neoplasias, anogenital, oro-laryngeal and -pharyngeal papillomas, keratoacanthomas and other types of hyperkeratoses. Their involvement in the etiology of some major human cancers is of particular interest: specific types (HPV 16, 18 and several others) have been identified as causative agents of at least 90% of cancers of the cervix and are also linked to more than 50% of other anogenital cancers. These HPV types are considered as '*high risk*' infections. Their E6/E7 oncoproteins stimulate cell proliferation by activating cyclins *E* and *A*, and interfere with the functions of the cellular proteins *RB* and *p53*. The latter interaction appears to be responsible for their mutagenic and aneuploidizing activity as an underlying principle for the progression of these HPV-containing lesions and the role of high risk HPV types as *solitary carcinogens*. In non-transformed human keratinocytes transcription and function of viral oncoproteins is controlled by intercellular and intracellular signalling cascades, their interruption emerges as a precondition for immortalization and malignant growth.

Recently, novel and known HPV types have also been identified in a high percentage of non-melanoma skin cancers (basal and squamous cell carcinomas). Similar to observations in patients with a rare hereditary condition, *epidermodysplasia verruciformis*, characterized by an extensive verrucosis and development of skin cancer, basal and squamous cell carcinomas develop preferentially in light-exposed sites. This could suggest an interaction between a physical carcinogen (UV-part of the sunlight) and a '*low-risk*' (non-mutagenic) papillomavirus infection. Reports on the presence of HPV infections in cancers of the oral cavity, the larynx, and the esophagus further emphasize the importance of this virus group as proven and suspected human carcinogens.

2. Introduction

The infectious nature of human and animal warts was demonstrated at the turn of this century (reviewed in [1]). First experimental attempts to relate these infections to cancer development and to study interactions with other carcinogenic factors were made by Rous and his associates in the 1930th and in the subsequent two decades [2–5]. Based on initial observations by [6], Rous demonstrated in ingenious experiments the carcinogenic potential of a cottontail rabbit papillomavirus infection in domestic rabbits and syncarcinogenic activity of tar and of defined chemical carcinogens, when jointly applied with the virus infection. The carcinogenic activity of the Shope papillomavirus (later renamed *cottontail rabbit papillomavirus* or *CRPV*) was subsequently irrefutably proven by [7], who induced carcinomas in domestic rabbits with purified CRPV DNA or with DNA extracted from CRPV-induced papillomas and carcinomas.

The first visualisation of papillomavirus particles in human warts by electronmicroscopy was reported in 1949 [8]. The structure of papillomavirus genomes was unravelled by Crawford and Crawford in 1963 [379]. The unavailability of tissue culture systems, however, and the apparent benign nature of human warts led to few additional experimental approaches in subsequent years.

Almost unnoticed by contemporary tumor virology, blossoming in the late 1950s and 1960s due to the discovery of murine leukemia viruses [9] and the DNA tumor viruses polyoma [10], SV40 [11], and adenovirus type 12 [12], two different lines of studies contributed to the development of papillomavirus research: In 1959 Olson and colleagues [396] reported the induction of urinary bladder tumors in cattle by a bovine papillomavirus found in skin fibropapillomas. The same virus turned out to be tumorigenic in hamsters [13,14] and transformed calf and

murine cells in tissue culture [15,16]. Thus, a second member of the papillomavirus group, besides CRPV, was clearly able to induce malignant tumors.

The second study roots back to 1922, when Lewandowsky and Lutz [392] reported a rare and obviously hereditary generalized verrucosis in humans with skin carcinoma development at sun-exposed sites. They labeled this syndrome *Epidermodysplasia verruciformis* and were not aware at that time of the potential infectious origin of the papillomatous plaques and macules covering the affected skin. This was subsequently demonstrated by [17] and by Jablonska and her colleagues [18,19] after inducing papillomas following intracutaneous autoinoculation of cell-free extracts. Jablonska realized in 1972 that this condition could serve '*as model in studies on the role of papovaviruses in oncogenesis*'.

Gradually interest in papillomaviruses evolved in the second part of the 1970s, evidenced by the first papillomavirus workshops, commonly attended at that time by 15 to 30 participants. This developed in part from the hypothesis that papillomaviruses may play a significant role in the etiology of cancer of the cervix [20,21]. Tests to substantiate this hypothesis had established the plurality of papillomavirus types and subtypes [22–24]. In addition, Meisels and Fortin [395] proposed a papillomavirus origin of koilocytotic atypias, separating them from 'true' preneoplastic lesions. This promised to represent a valuable diagnostic aid in grading lesions for surgical intervention. The demonstration of papillomavirus particles in typical koilocytes underlined their observations [25–27]. Papillomavirus research, however, was also stimulated by the identification of novel HPV types in lesions of patients with epidermodysplasia verruciformis (EV), and here particularly in malignant tumors of such patients [24,28].

In the 1980s the situation changed almost abruptly: the isolation of new HPV types (HPV 6 and 11) from genital warts [29,30], and subsequently directly from cervical cancer biopsies HPV types 16 and 18 [31,32] resulted in a rapid expansion of experimental work and also in early epidemiological approaches. In spite of numerous efforts from various laboratories, it took almost one decade before the causal role of specific HPV types in cancer of the cervix and the respective precursor lesions was more or less generally accepted [199,33–35].

Today the main interest shifted to mechanisms of carcinogenesis by papillomaviruses: how do genes of these viruses influence cell growth, how do their oncoproteins interact with host cell components, and to which extent is the failure of specific host cell functions related to papillomavirus-induced oncogenesis? The very recent recognition of a linkage of other widespread human tumor types, like cancers of the skin and of the oropharynx, with HPV infections points to the magnitude of the problem. Papillomaviruses emerge as the most common *carcinoma viruses* [36] and appear to play a 'secret' role as major cancer pathogens [37]. Successful first attempts to vaccinate animals against their own papillomavirus infections [377], raise at the same time the hope for the prevention of specific human cancers based on similar vaccination protocols.

This review tries to summarize our present understanding of papillomavirus infections by emphasizing their role in human cancers and by analyzing specific aspects of their interaction with the infected host and specific host cell components. For a detailed description of the molecular biology of papillomaviruses and of immunological and epidemiological aspects the reader is referred to a number of other reviews [402,38,384,219,388].

3. Structure of viral particles and taxonomy

3.1. Viral particles and late proteins

The diameter of papillomavirus (HPV) particles amounts to approx. 55 nm. Full particles contain the double-stranded closed circular DNA genome. The viral DNA is associated with histone-like proteins [39,40] and encapsidated by 72 capsomeres [41]. The major capsid protein is coded for by the L1 open reading frame, it seems to contain reactive epitopes for type-specific neutralization. The L2 open reading frame codes for an additional structural component of the viral capsid. Antigenic domains of this protein appear to be responsible for a group-specific reactivity of antisera. Virus-like particles, containing the structural components of various types of HPV can, however, be obtained by the expression of these proteins in recombinant vectors [42,43], obviously the L1 protein suffices for this particle formation. This protein has a mol wt. of approx. 55 000 and is highly conserved among different papillomavirus types. The second structural protein, L2, is less conserved and possesses a molecular weight of about 75 000. The non-enveloped structure renders papillomaviruses relatively resistant to heating and to organic solvents [44].

3.2. Structure and regulation of the viral genome

The genome consists of 7200–8000 base pairs of closed-circular double-stranded DNA containing up to 10 open reading frames (Fig. 1). The structure of the viral genomes reveals remarkable similarities between different members of this virus group: generally only one strand is transcriptionally active, therefore transcription occurs in one direction only, and the localization of open reading frames reveals a remarkable degree of correspondence [45,46]. Papillomavirus genomes can be divided into three regions: a long control region (LCR) covering about 10% of the genome, and early (E) and a late (L) region. The L genes code for structural proteins, the E region mainly for regulatory functions engaged in genome persistence, DNA replication, and activation of the lytic cycle.

The regulation of viral gene expression is complex and controlled by cellular and viral transcription factors. Most

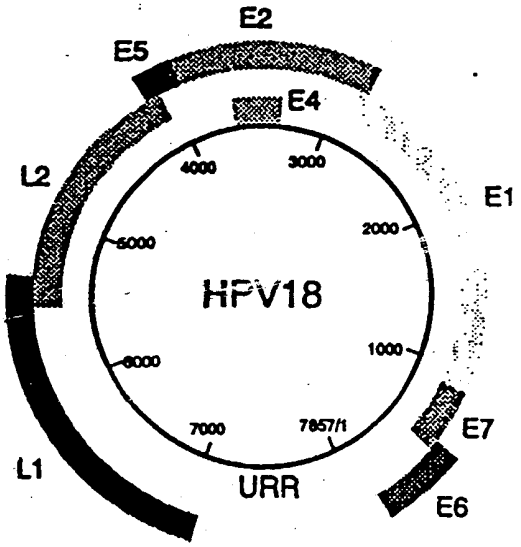

Fig. 1. Circular map of the HPV18 genome indicating the localization of open reading frames (E1 to E7 for early proteins, L1 and L2 for late proteins) and the upstream regulatory region (*URR* or long control region, *LCR*). The figure was kindly provided by Dr. Felix Hoppe-Seyler.

of these regulations occur within the LCR region which varies substantially in nucleotide composition between individual HPV types. The LCRs of anogenital HPVs range in size between 800–900 bp, in other papillomaviruses, particularly in those found in EV-lesions, they are somewhat shorter. Within the LCR *cis*-active elements regulate transcription of the E6/E7 genes which represent the transforming genes for immortalization and for the maintenance of the malignant phenotype of HPV-positive cervical cancer cells [47–49].

A large number of cellular transcription factors have been identified, binding to the frequently studied HPV18 LCR: among them NF-1, AP1, KRF-1, Oct-1, SP-1, YY-1, and the glucocorticoid receptor [50–52,409,54, 410,411,55,56]. The dysfunction of some of them appears to play a significant role in papillomavirus-linked carcinogenesis [57]. Very recently a novel epithelial factor, Epoc-1/Skn-1a, was identified, regulating papillomavirus transcription differentiation-dependent in suprabasal cells [58]. Most of these factors bind to the central region of the LCR, the *enhancer*-region. They regulate the transcription of the E6/E7 promoter located at the 3'-terminus of the LCR. Although many of these factors stimulate the promoter, some of them (specifically YY-1) have a dual function and repress and stimulate the viral promoter [56,59,282].

Besides the cellular regulation, intragenomic regulation of the E6/E7 gene activity by the viral E2 protein plays an important role in activating or suppressing these oncogenes (see below). The LCR of HPV 16 contains four E2 binding sites. The 5'-distal part of the LCR contains the first E2 binding site, forming the terminal part of this region, and the translation termination codon for L1. The same segment harbours transcription termination and polyadenylation sites for late transcripts and acts as a negative regulator at the level of late mRNA stability [60,61].

The central segment of the LCR is flanked by two E2 binding sites. The E2 binding site at the 3' part is engaged in replication initiation, but also modulates E6/E7 transcription [55]. The proximal segment contains the promoter region, terminated 5' by the E2 site and 3' by the translation start codon of the E6 gene. Two additional E2 binding sites are located within the proximal 90 base pairs overlapping a TATA box. E2 binding of these sites modulates the promoter activity by displacing the basic transcription complex [381,62].

3.3. Nomenclature and taxonomy

Since 1976 [22], the genetic heterogeneity of the human papillomavirus group became more and more apparent. In 1978 a small conference took place in Mobile, Alabama, resulting in a proposal for HPV nomenclature: it was decided to designate new types if they differed by more than 50% from known prototypes when tested by reassociation kinetics performed under stringent conditions of hybridization [63]. When more and more sequence data became available, it was decided on one of the subsequent workshops to use DNA comparisons of the E6, E7, and L1 open reading frames for typing of HPVs [64]. An overall difference within these open reading frames of more than 10% was used to define new types. At the Annual Papillomavirus Conference 1995 in Quebec City this issue was reconsidered. Differences in only the L1 open reading frame exceeding 10% from established prototypes were used from now on for the definition of new types.

To date 77 distinct HPV genotypes have been described and the genomic sequences of most of them have been fully analyzed [65,380]. They are listed in Table 1. About 30 additional partial sequences have been obtained of putative novel HPV types, suggesting that the total number of existing HPV genotypes exceeds well 100. The explosion in identifying novel types originates from technical advances, particularly from the application of PCR technology. The arbitrary definition of novel types, a difference of more than 10% in the nucleotide sequences in the E6, E7, and L1 open reading frames, seems to define natural taxonomic units, since most recent isolates represented either novel types or were identical or differed only marginally from established prototypes [66]. In spite of the enormous heterogeneity of this virus group, mutational changes appear to occur at low frequency, indicating diversification of the types already in prehistoric times.

Based on their nucleic acid composition, a number of papillomavirus subgroups can be defined [67]. One of the largest known subgroup is represented by HPV types infecting mainly mucosal surfaces, most frequently of the anogenital tract. More than 40 of the identified HPV types belong into this group. The best known prototype of this

Table 1
Characterized HPV types (from [64,65], and unpublished data)

HPV type	Preferentially found in:
1	plantar warts
2	common warts
3	flat warts
4	common warts
5	benign and malignant EV lesions
6	genital warts, laryngeal papillomatosis
7	'butcher's' warts, oral papillomas of HIV patients
8	benign and malignant EV lesions
9	EV lesions
10	flat warts
11	laryngeal papillomas, genital warts
12	EV lesions
13	oral focal epithelial hyperplasia
14	EV lesions
15	EV lesions
16	anogenital intraepithelial neoplasias and cancers
17	EV lesions
18	anogenital intraepithelial neoplasias and cancers
19	EV lesions
20	EV lesions
21	EV lesions
22	EV lesion
23	EV lesions
24	EV lesions
25	EV lesions
26	common warts under immunosuppression
27	common warts
28	flat wart
29	common wart
30	laryngeal carcinoma
31	anogenital intraepithelial neoplasias and cancers
32	oral focal epithelial hyperplasia, oral papillomas
33	anogenital intraepithelial neoplasias and cancers
34	anogenital intraepithelial neoplasias
35	anogenital neoplasias and cancers
36	actinic keratosis, EV lesions
37	keratoacanthoma *
38	melanoma *
39	anogenital intraepithelial neoplasias and cancers
40	anogenital intraepithelial neoplasias
41	cutaneous squamous cell carcinomas
42	anogenital intraepithelial neoplasias
43	anogenital intraepithelial neoplasias
44	anogenital intraepithelial neoplasias
45	anogenital intraepithelial neoplasias and cancers
46 **	EV lesions
47	EV lesions
48	cutaneous squamous cell carcinoma
49	flat wart under immunosuppression
50	EV lesion
51	anogenital intraepithelial neoplasias and cancers
52	anogenital intraepithelial neoplasias and cancers
53	anogenital intraepithelial neoplasias
54	anogenital intraepithelial neoplasias
55	anogenital intraepithelial neoplasia
56	anogenital intraepithelial neoplasias and cancers
57	oral papillomas and inverted maxillary sinus papilloma
58	anogenital intraepithelial neoplasias and cancers
59	anogenital intraepithelila neoplasias
60	epidermoid cysts
61	anogenital intraepithelial neoplasias
62	anogenital intraepithelial neoplasias
63	myrmecia wart
64	anogenital intraepithelila neoplasia
65	pigmented wart
66	cervical carcinoma
67	anogenital intraepithelial neoplasia
68	anogenital intraepithelial neoplasia
69	anogenital intraepithelial neoplasias and cancers
70	vulvar papilloma
71	anigenital intraepithelila neoplasia
72	oral papilloma (HIV patient)
73	oral papilloma (HIV patient)
74	anogenital intraepithelial neoplasia
75	common wart in organ allograft recipient
76	common wart in organ allograft recipient
77	common wart in organ allograft recipient

* Only individual isolates.
** Now designated HPV 20b.

group is HPV16. Another subgroup is represented by viruses found in epidermodysplasia verruciformis lesions, with HPV5 as the most prominent member. These viruses are also found in lesions of patients suffering from immunosuppression. A third subgroup contains a few virus types preferentially found in cutaneous lesions. The prototype of this subgroup is HPV 4. A fourth subgroup finally is in itself heterogeneous and contains some distantly related viruses like HPV1, HPV63, and HPV41.

The heterogeneity of the human papillomavirus group is not restricted to the human members, the large number here seems to reflect the intensity of investigations. Thus far 8 bovine papillomavirus types have been isolated. Four types have been cloned from monkeys and apes.

It is interesting to note that a number of animal papillomaviruses are more closely related to individual members of the human subgroups than the latter among each other. A rhesus monkey papillomavirus isolated from a penile carcinoma of these monkeys [68] is very closely related to HPV 52. Similarly, there exists a very close relationship between a pygmy chimpanzee papillomavirus and HPV13 [69]. The cottontail rabbit papillomavirus and the canine oral papillomavirus belongs into the subgroup of HPV1, 63, and 41 [70,71]. These observations stress the assumption of the development of the papillomavirus group far back in prehistoric times.

4. Functions of viral proteins

4.1. E2 protein

The E2 open reading frame encodes at least two and probably three different proteins, all acting as transcription factors [72]. They differently affect viral gene expression and represent major intragenomic regulators by forming

dimers at specific binding sites. HPV16 and HPV 18 E2 protein function as transcriptional activators in human cervical keratinocytes [72–74]. The C-terminal domain of the HPV 16 E2 gene acts as transcriptional repressor and interferes with the activity of the full length E2 protein [75].

Deletion of the E2 open reading frame is frequently observed in cervical cancer biopsies and in cell lines derived from this cancer [76], leading to the speculation that this deletion facilitates transformation of human cells and the transition into a malignant state. Indeed, mutations in the E2 ORF, but also in the E2 DNA binding sites within the viral LCR led to enhanced immortalizing activity of HPV 16 DNA [77]. In cancer development, disruption of E2 appears, however, to usually represent a late event since most premalignant lesions do not reveal this modification [78,79]. A recent study noted integration of HPV 16 DNA also in advanced cervical intraepithelial neoplasias [80]. Besides its role in transcriptional regulation, E2 proteins interacting with E1 stimulate viral DNA replication [81–83]. They apparently facilitate binding of E1 to the origin of replication [84].

4.2. E1 protein

E1 shares a number of properties with SV40 large T antigen [84,85]: It codes for a policistronic RNA, the protein has site-specific DNA binding functions [86], binds and hydrolyzes ATP [84], possesses ATP-dependent helicase activity [87] and is essential for papillomavirus replication [88]. It also interacts with cellular DNA polymerase α [407]. The E1 protein binding site in the origin of replication, localized in the proximal region of the LCR, represents an 18 nucleotides imperfect palindrome [89]. Bidirectional unwinding of this region is a prerequisite for viral DNA replication [90]. Besides L1, the E1 open reading frame represents the most conserved structure among different papillomavirus types.

4.3. E5 protein

The E5 protein is the major transforming protein in bovine papillomaviruses [91–93]. In contrast, in HPV infections E5 has only weak transforming activity [94–96]. It may cause tumorigenic transformation of mouse keratinocytes, leads to anchorage-independent growth of mouse fibroblasts, and stimulates growth of primary rat kidney epithelial cells in cooperation with the HPV16 E7 gene [97,72]. The open reading frame coding for E5 is frequently deleted in cervical cancers [76], although anogenital low grade intraepithelial neoplasias contain relatively large amounts of E5 mRNA and protein [98,99]. This may support the assumption that E5 plays a role in early steps of HPV infection but is obviously dispensable for the maintenance of malignant transformation.

The hydrophobic E5 protein is mainly localized within the Golgi apparatus, in part it is also found in the plasma membranes [100]. BPV1 E5 protein binds and enhances the effect of platelet-derived growth factor (PDGF) and of epidermal growth factor (EGF), an effect not seen by HPV 16 E5 [101–103]. The latter E5 protein, however, reduced the degradation of internalized EGF receptors. Recent studies demonstrate complex formation between HPV 16 E5 protein with epidermal growth factor receptor, platelet-derived growth factor β receptor, colony stimulating factor-1 receptor, and with vesicular stomatitis virus glycoprotein [104]. Thus, this protein complexes with a variety of other transmembrane proteins. BPV1 and HPV16 E5 proteins also associate with the membrane-bound proton-ATPase which is part of the gap-junction complex [105,383]. In HPV16 E5-transfected cells a strong impairment of microinjected Lucifer yellow was noted, correlating with dephosphorylation of connexin 43, a major gap junctional protein [106].

4.4. E4 protein

The E4 protein seems to be incorrectly assigned as an early gene product. It originates from a viral RNA transcript formed by a single splice between the beginning of the E1 open reading frame and the E4 open reading frame. This mRNA is the major transcript in HPV-induced lesions [107,108]. The role of this protein in the life cycle of the virus has yet to be determined. It is not required for transformation or episomal persistence of viral DNA [109]. The E4 protein is exclusively localized within the differentiating layer of the infected epithelium [110,111,325,397]. It has been speculated that this protein plays a role in productive infection, possibly by disrupting normal differentiation, establishing favorable conditions for viral maturation.

E4 proteins associate with the keratin cytoskeleton of cultured epithelial cells [112,113]. Electron microscopically they can be localized to tonofilament-like structures in HPV1 warts [412]. HPV16 E4 induces a collapse of the cytokeratin network in cultured cells [112,113]. Multiple E4 proteins have been demonstrated in HPV1-infected cells [110]. This may result from differential expression but also from posttranslational modifications and should influence the functional activity of E4 proteins [114].

Even papillomavirus types sharing tissue specificity reveal only limited homology in DNA sequences coding for E4 proteins [115]. The HPV1 E4 protein has been identified as a zinc finger protein [116]. The functional consequences of this property are presently unknown.

4.5. E6 and E7 proteins

E6 and E7 proteins are expressed in HPV-positive cancer cells These proteins may cause immortalization of

human keratinocytes and of a number of other cell types (see Section 4). Those HPV types coding for E6 and E7 genes involved in immortalization of tissue culture cells and found frequently in malignant tumors, are considered as *high risk* HPVs, contrasting an apparently low tumorigenic potential of other types, generally designated as *low risk* HPVs [117]. E6, and E7 genes code for growth-stimulating proteins, particularly E6 and E7 of specific types are relevant for the progression to malignant growth (reviewed in [118]).

Both proteins of high risk types cooperate in immortalization and transformation [408,47]. E7 proteins of these viruses, however, are able to transform established rodent cell lines by themselves (reviewed in [119]. Similarly E6 represents an independent oncogene since it immortalizes human mammary epithelial cells [120].

4.5.1. E6 protein

The E6 protein of HPV 16 contains 151 amino acids and reveals four Cys-X-X-Cys motifs mediating zinc-binding which may result in the formation of two zinc finger structures [121–123]. The E6 protein of high risk HPVs possesses a number of interesting biological properties: it cooperates with the E7 protein in the immortalization of human cells [47]. Introduction of this gene into specific types of human mammary cells may lead to immortalization even in the absence of E7 [120,124]. E6 of these virus types, in addition, cooperates with the *ras* oncogene in the immortalization of primary rodent cells [125] and induces anchorage-independent growth of NIH 3T3 cells and transcriptionally transactivates the adenovirus E2 promoter [126].

A most significant observation related to the function of the E6 protein was made initially by [127], revealing the binding of the cellular p53 protein to E6. This was followed by experiments showing that this binding promotes the degradation of p53 [128] mediated by the cellular ubiquitin proteolysis system [129,385]. p53 acts as an transcriptional activator by binding to specific DNA sequences [130] and is required for the growth arrest following cellular DNA damage [131,132]. Cells without functioning p53 are not arrested appropriately in G1 and display genomic instability [133,134]. The transcriptional activation by p53 induced after DNA damage is inhibited by HPV18 E6 [135].

The interaction of E6 with p53 is obviously the prime cause of chromosomal instability in cells infected by high risk HPVs [136–138]. It sensitizes human mammary epithelial cells to apoptosis induced by DNA damage [139]. In addition, the abrogation of the p53 function by transfection with the HPV16 E6 gene enhances the resistance of human diploid fibroblasts to ionizing radiation [140].

Degradation of p53 by E6, though apparently responsible for chromosomal instability and therefore presumably one of the main risk factors in the progression of premalignant lesions (see below), is not the sole function of this viral oncoprotein. The degradation of p53 seems not to be sufficient for a growth-stimulatory effect of E6 observed in human embryonic fibroblasts [141]. Moreover, the E6 protein of HPV16 functions as a transcriptional repressor of the Moloney murine leukemia virus long terminal repeat and of the cytomegalovirus immediate early promoter [142].

In addition to binding p53 and the cellular ubiquitin E6-AP, E6 interacts with various other, yet poorly defined cellular proteins [143] and with a putative calcium-binding protein [144].

4.5.2. E7 protein

The HPV16 E7 protein represents a zinc binding phosphoprotein with two Cys-X-X-Cys domains composed of 98 amino acids. A zinc binding domain and two Cys-X-X-Cys motifs reveal similarity to the E6 protein, suggesting an evolutionary relationship between the two proteins. The amino terminal part of the E7 protein contains two domains corresponding partially to the conserved region 1 (CR-1) and completely to conserved region 2 (CR-2) of adenovirus E1A proteins and to an analogous region in SV40 large T antigen [145]. Both of the E1A regions are involved in cell transformation [146]. Both corresponding domains in E7 (cd-1 and cd-2) contribute to the immortalizing potential of E7 [147]. In a yeast two-hybrid system dimerization of the E7 oncoprotein has been demonstrated in vivo [414].

Similar to E1A and SV40 large T antigen, high risk HPV E7 proteins complex with the retinoblastoma susceptibility protein pRB [148–150]. The binding affinity of high risk HPVs E7 for pRB is approx. 10-fold higher than that of low risk HPVs [384]. This difference results apparently from a single amino acid modification at the position 21 [151] which also influences the ability of E7 to cooperate with an activated *ras* gene in transformation of baby rat kidney cells [152]. pRB-binding, however, does not emerge as a general precondition for immortalization [153], pointing to additional functions of the E7 protein. In correspondence to E1A/pRB complexes, E7/pRB binding releases the transcription factor E2F from pRB complexes, activating transcription of genes regulating cell proliferation [154,155]. Interestingly, a strong RB-binding activity has also been reported for the low risk HPV1 virus E7 protein [156] which fails, however, to reveal other transactivating activities.

Besides binding pRB, E7 proteins of high risk viruses associate with related proteins, such as p107 and p130, and with the protein kinase p33cdk2 and with cyclin A [157,158]. Recent studies demonstrate that E7 expression in NIH3T3 cells results in a constitutive expression of cyclin E and cyclin A genes in the absence of external growth factors [159]. E7 activates the cyclin A promoter via an E2F binding site. Cyclin E activation requires the cd-2 domain, but not cd-1, whereas cyclin A activation

requires both domains. The cyclin E activation precedes that of cyclin A. Obviously E7 overrides two inhibitory functions restricting expression of cyclin E and cyclin A genes. Cyclin D1 expression is not affected by E7. The analysis of E7 mutants indicates that the activation of cyclins E and A cosegregates with the ability of E7 to transform [159]. Similarly, transcriptional activation by HPV16 E7 has also been reported from the adenovirus E2 [160,161] and B-myb promoters [162].

Recently it has been demonstrated that the HPV16 E7 protein can complement functions of E1A provided by the E1A aminoterminus and required for stimulation of adenovirus type 5 early promoters [163]. Under these conditions the association of protein complexes containing c-jun with ATF sites is stabilized. In addition, by using a glutathione-S-transferase fusion protein system, E7 complexes with AP-1 transcription factors have been demonstrated including *c-jun*, *jun-B*, *jun-D* and *c-fos* [415]. Mutational analysis revealed that the E7 zinc-finger motif, but not the pRB binding domain were involved in these interactions. Since a transcriptionally inactive *c-jun* deletion mutant also bound E7 and interfered with E7-induced immortalization, the *jun*-E7 interaction appears to be physiologically relevant.

High risk E7 proteins, to a lesser extent than E6 proteins (see below), can bypass DNA-damage p53-induced G_1 growth arrest [164–166,136], as a potential mechanism for the reported E7-induction of chromosomal aberrations [167]. The mode of this interaction is presently not fully understood, but suggests an interconnection between p53 and RB regulating pathways.

5. Transmission and natural history of papillomavirus infections

Transmission of human papillomaviruses is facilitated by the presence of abraded or macerated epithelial surfaces [168]. Anogenital infections are mainly transmitted by sexual contact. HPV DNA is rarely detected in sexually unexperienced young women [169–172]. There exists a correlation between the number of sexual partners and the prevalence of HPV infection [173–176]. Occasionally, anogenital HPV infections are also transmitted digitally from one epithelial site to the other [177,382]. They may be transmitted by fomites, by medical instruments and by laser plumes [178,179].

Oral-genital contact may lead to infections at oral sites by anogenital HPVs [180]. Salivary transmission probably accounts for additional infections of this region.

Skin infections by papillomaviruses originate from contacts with contaminated materials, walking barefoot on an abrasive surface [181,182] or by acquiring accidental epithelial wounding with contaminated equipment [183].

The natural history of HPV infections is presently not fully understood. It appears that the majority of these infections does not lead to visible lesions, it may be in fact abortive or is cleared by the immune system within a short period of time. Indeed, severe impairment of immune functions results in a high prevalence of clinically apparent HPV infections [184–187]. Obviously, infections with HPVs found almost exclusively in a rare hereditary condition, epidermodysplasia verruciformis (EV) (see below), must also be spread within the non-EV-population, since

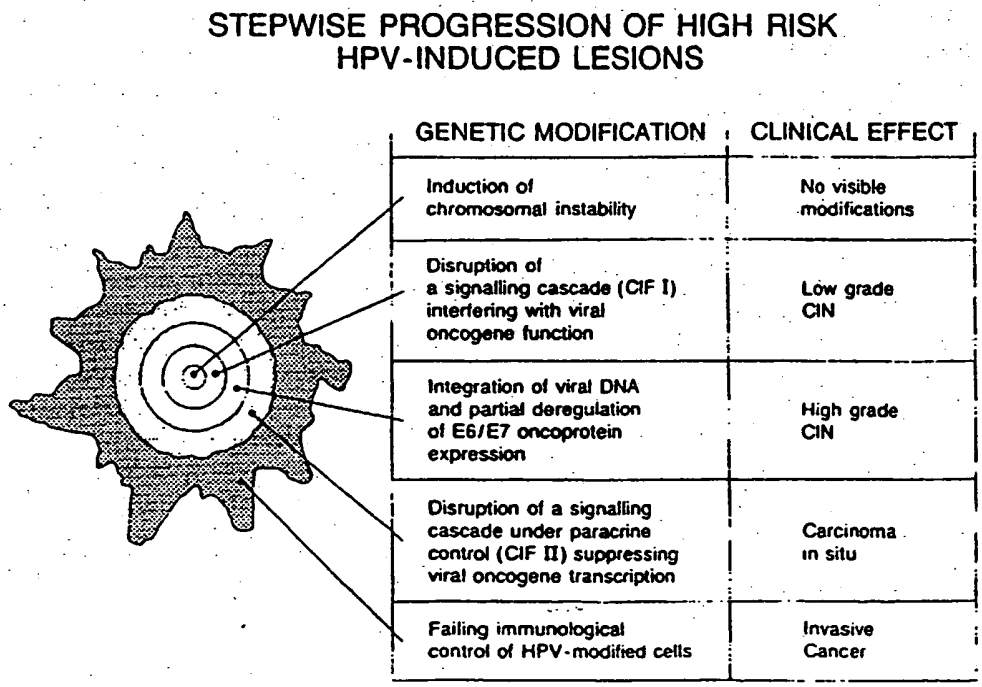

Fig. 2. Stepwise progression of high risk HPV-induced lesions. The inner circle schematically represents initially infected cells, subsequent circles symbolize progressively modified clones with increased expansion and growth potential.

individual cases of EV have been noted worldwide (see review [188]).

An important, yet unresolved issue is the clonality of intraepithelial lesions and warts. The existing literature on this topic is scarce and controversial [189,190]. A clonal growth of HPV-infected tissue may suggest that the development of lesions required already a highly specific intracellular environment, possibly only provided in cells with specific genetic modifications (selective gene inactivations or activations, mutations).

For anogenital HPV infections a schematic view of the progression of lesions is depicted in Fig. 2, mainly based on experimental findings within various stages of progression. The percentage of initially high risk HPV-infected women developing cancer of the cervix at a later stage of life is low [191]. It should be still much lower in infections with low risk viruses.

Virtually nothing is known about the mode of viral genome persistence in unapparent, latent, infections. HPV DNA presence has been demonstrated in clinically symptom-free epidermal and mucosal sites of the cervix, the larynx or the skin [192–195]. The mode of maintenance of this DNA persistence is unknown.

6. Non-malignant proliferations induced by papillomavirus infections

Papillomaviruses cause a wide spectrum of cutaneous, mucocutaneous and mucosal proliferations (see [196]). There exist various forms of common warts with an interesting localization-specific pattern of virus-types: plantar warts contain, for instance, most frequently HPV1 [24], the myrmecia type of plantar warts is frequently linked with HPV 63 infections [197], hand warts contain most often HPV2 and HPV4 [23,24]. It appears that individual virus types induce a somewhat specific histopathology and differ in their growth-stimulating potential.

The wart, however, is not the sole cutaneous manifestation of infections by specific types of papillomaviruses: various HPV types have also been noted in Bowenoid lesions and other intraepithelial neoplasias [177,198]. Again, the development of premalignant changes regularly, though not exclusively, depends on the infecting virus type. Solar exposure of HPV infected sites or HPV infection of the sun-exposed skin may lead to syncarcinogenic effects even after infection with low risk viruses [198].

Anogenital HPV infections are found in either mucocutaneous or mucosal localizations. It appears that HPV6 and HPV11 which cause the vast majority of genital warts prefer mucocutaneous sites, since most active proliferation and virus production is noted in condylomata acuminata at external genital sites. These viruses are only rarely found in cervical infections. HPV16, less frequently HPV18, and several other anogenital HPV types which probably represent high risk infections, cause Bowenoid lesions at external genital and perianal sites (vulvar, penile, perianal and anal intraepithelial neoplasias). The same viruses, besides some additional low risk HPV infections, are responsible for cervical intraepithelial neoplasias [199]. A careful analysis recently revealed that more than 96% of these lesions contain identifiable HPV types [35].

Oral lesions may contain anogenital HPV types, very regularly HPV11 or HPV6 are found in laryngeal papillomatosis [30]. Oral papillomas frequently contain these or other anogenital types [64,200,201]. Very few types have been thus far exclusively detected in the oral mucosa, besides HPV13 [202] and HPV32 [203] two new types were recently isolated from oral lesions of immunosuppressed patients, HPV 72 and HPV 73 [201].

It is likely that the vast majority of HPV infections remains clinically without symptoms or produces unapparent microlesions. The wide distribution of HPV types throughout the world, even of those found only in rare clinical conditions, like epidermodysplasia verruciformis, strongly suggests an effective mode of spreading within the human population and probably long periods of persistence and virus particle shedding from infected individuals. Though not stringent, there seems to exist a certain degree of specialization of subsets of HPV types to specifically differentiated human cells which either permit efficient infection or provide optimal conditions for viral particle maturation. The adaptation to these tissues may in part explain the development of the surprising heterogeneity of the papillomavirus group in the course of evolution within the same host.

7. Papillomaviruses causing cancer: general considerations and mechanistic aspects:

7.1. General considerations of causality

Although an infectious etiology of cancer has been suspected by some investigators for more than one century (reviewed in [204], and first animal data on cancer induction by animal viruses became available approximately 90 years ago [205,206], it proved to be exceedingly difficult to demonstrate a causal involvement of infections in human cancers. Several reasons account for this difficulty [207]:

Infections suspected to be involved in human cancerogenesis are frequently ubiquitous (e.g., Epstein-Barr virus, papillomaviruses, hepatitis viruses). Only a small percentage of infected individuals develops the respective form of cancer.

The time periods elapsing between primary infection and cancer development are frequently in the order of several decades.

The arising tumors are commonly monoclonal and, thus, cannot be the result of a systemic infection.

Chemical and physical cancerogens are frequently suspected to be causally related to the same tumor types.

These notions appear to be incompatible with cancer development as the immediate or sole consequence of an infection. The infection still could be necessary, but should be not sufficient for cancer induction.

One additional aspect adds to these difficulties: viruses may contribute to cancerogenesis by very different modes of interaction. Besides viral infections which seem to exert a growth-stimulating effect by *direct* and continued interactions with infected host cells (e.g. viral genome latency with continued expression of viral oncogenes, *cis*-activation of cellular genes due to viral DNA integration), other infections contribute to cancerogenesis *indirectly* (reviewed in [208]. Human immunodeficiency viruses (HIV) substantially elevate the risk for specific cancers (B-cell lymphomas, Kaposi sarcomas, non-melanoma skin cancers). Other virus infections, particularly with members of the herpes virus group, induce mutations in host cell DNA even after abortive and transient infections [209] and amplify under the same conditions the DNA of other DNA tumor viruses persisting in the same cells [210–212].

Papillomaviruses infections contribute directly to carcinogenesis, since (with the exception of one animal system [213]) viral DNA persists in the malignant cells and is genetically active [32,76]. Therefore, the following discussion will be limited to the potential causal link between these infections and human cancers and to *trans*-functions involved in cancer development.

In conventional infections Koch's postulates [214], with minor modifications, have been used effectively to establish causality. The propagation of the suspicious agent under experimental conditions outside the host and the induction of the respective disease in suitable experimental animals after prior in vitro cultivation are not applicable to many suspected human tumor viruses. Most of those cannot be propagated under experimental conditions nor are they tumorigenic for laboratory animals.

To overcome these problems, Evans [215] stressed seroepidemiological data as additional parameter to establish relationship between virus infections and specific human cancers. His suggestions were motivated by seroepidemiological data linking Epstein-Barr virus to specific human cancers (see review [216]). In papillomavirus infections none of these parameters holds up as valid: besides the absence of effective in vitro replication systems and suitable animal hosts, even seroepidemiology provides insufficient information. A substantial percentage of cervical cancer patients carrying HPV16- or 18-positive tumors appears to be devoid of detectable immune responses to antigens of the respective virus [217,218]. The now emerging possibility of seroepidemiological tests by using virus-like particles [42,43] may permit a more detailed analysis of the immunological response against HPV infections.

In spite of these problems, are there criteria available permitting an unequivocal establishment of causality for these infections? Indeed, this has been attempted previously [219] and is summarized as follows:

Epidemiological evidence (risk assessment, coincidence of geographic prevalence, seroepidemiology, plausibility of relationship) that the respective infections represent risk factors for the development of specific tumors;

Regular presence and persistence of nucleic acid of the respective infectious agent in cells of specific malignant tumors;

Stimulation of proliferation upon transfection of the respective genome or parts thereof in corresponding tissue culture cells;

Demonstration that the induction of proliferation and the malignant phenotype of specific tumor cells depend on effects or functions exerted by the persisting DNA of the infectious agent.

The most convincing criterium originates from experiments where the genetic activity of the latent viral genome has been knocked out in cervical carcinoma cells. As the consequence, a decreased proliferation rate and loss of the malignant phenotype have been demonstrated [49,220,221].

The application of these criteria appears to be useful for those viral systems where position effects of viral DNA integration or specific viral gene functions are suspected to be involved in cancerogenesis.

7.2. High and low risk HPVs

The original definition of specific HPV types as high risk viruses was based on their frequent presence in cervical and anogenital cancers [117]. In subsequent years the assignment of different properties to both groups of agents permitted a focussing of this definition. This became apparent when 'high risk' viruses were shown to immortalize human keratinocytes [222,223], whereas low risk viruses failed to do so. The subsequent observations on p53 and pRB binding by high risk HPV oncoproteins [127,150], in remarkable contrast to several low risk viruses, seemed to contribute another functional parameter for this differentiation. The induction of chromosomal aberrations as the consequence of high risk viral oncoproteins overriding cell cycle control mechanisms [224,164–166,136] emerges as the functionally most important distinction between these virus groups. Due to these properties high risk viruses are able to directly contribute to the progression of latently infected cells and may act as solitary carcinogens [208].

Cancer cells containing low risk HPV genomes frequently reveal modifications in the cellular p53 gene and occur at sites exposed to chemical or physical carcinogenic factors. Basal cell and squamous cell carcinomas of the skin (see below) are one example, extensive laryngeal papillomatosis which had been X-irradiated in past decades and converted subsequently into squamous cell carcinoma of the larynx (summarized in [405]) represent another one. Although the actual role of low risk HPVs in these malignant conversions is not yet clarified, the observations

suggest that mutagenic modifications of host cell genes, presumably required to activate the oncogenic potential of these viruses, are mediated in these instances by the physical carcinogens. The apparent inability of these viruses to code for mutagenic oncoproteins seems to be the main reason for their failure to act as solitary carcinogens and for their dependence on interaction with other mutagenic factors in the generally rare events of malignant development following these infections.

8. Immortalization of tissue culture cells by papillomaviruses

Immortalization of tissue culture cells by viruses is defined by the induction of continuous growth in vitro without detectable tumorigenicity of these cells after heterotransplantation into immunosuppressed animals. Transformation, in contrast, defines continuous growth of cells which, upon heterotransplantation under the same conditions, form invasively growing tumors.

The definition of immortalization is derived from experimental conditions. It is not entirely clear how it correlates to clinical HPV-induced lesions. It is, however, suggestive that it corresponds at least to a proportion of low grade intraepithelial neoplasias, as deduced from three types of observations: immortalized cells in organotypic cultures histologically resemble low grade intraepithelial lesions (reviewed in [225]. In addition, clinical low grade neoplasias, in contrast to high grade lesions and invasive cancer, reveal a similar restriction of E6/E7 oncogene transcription, as do immortalized cells, when the latter are heterografted into immunocompromised animals [79]. Finally, though difficult, it has been possible to cultivate immortalized lines from explants of intraepithelial neoplasias [226–228].

Early attempts to immortalize cells by papillomavirus infection date back to 1963 when Black et al. and Thomas et al. demonstrated immortalization of fetal bovine cells by bovine papillomavirus infection. In 1980 Lowy and colleagues [393] showed that only 69% of the genome was required for successful immortalization.

The first reports on attempts to immortalize or transform murine cell by human papillomavirus types appeared in 1984 and 1986 [229,230]. This was quickly followed by similar data after transfection of rat cells with HPV16 or HPV18 DNA [231,232]. At about the same time it became apparent that the HPV16 E7 gene cooperates with the *ras* oncogene in the transformation of primary rat kidney cells [233,161].

Immortalization of human cells was with HPV16 DNA was first achieved in 1987 [222,223] and with HPV18 DNA in 1988 [237]. Subsequently a large number of additional human cell types, including skin, bronchial and kidney epithelium, smooth muscle and endothelial cells, have been immortalized by high risk HPV DNA transfection (reviewed in [225]). Recent reports describe the immortalization of human prostate [234] and ovarian cells [235] by high risk HPV DNA. Only DNA fragments carrying the E6/E7 genes were necessary for immortalization of rodent [231] and human cells [236,237,47].

Although the expression of E6/E7 genes is necessary for immortalization of cells by high risk HPV, this expression is clearly not sufficient [238]. There exists good evidence for the need of modifications in specific host cell genes as an additional prerequisite for immortalization. This has been demonstrated by somatic cell hybridization studies, initially performed with cells from other virally immortalized lines [239,242,240], subsequently also with HPV-immortalized cells [241]. Only a small fraction of initially infected or transfected cells eventually becomes immortalized, the vast majority of these cells continues to express viral oncoproteins [242] or, in the case of HPV transfection, continues to transcribe E6/E7 message [241] and undergoes senescence.

Somatic cell hybridization performed with different clones of immortalized cells led to the identification of four complementation groups complementing each other for senescence. Although this number may increase in the future, it suggests that the failure of each one of at least four cellular genes, presumably engaged in the regulation of the same signalling pathway [57], in addition to viral E6/E7 gene expression may result in immortalization.

The involvement of cellular genes whose failing function in the presence of HPV oncogenes leads to immortalization points to two predictions: the function of these genes in non-modified cells interferes with the function of viral oncoproteins, as evidenced by the continued expression of the latter even in cells undergoing senescence. Secondly it leads to the expectation of specific mutational changes, possibly even visualized by specific chromosomal aberrations, in immortalized cells. There exist indeed first reports on specific chromosomal aberrations in HPV-immortalized human keratinocytes, preferentially involving sites on chromosomes 3 and 18 [243–245,390]. The interpretation of these data is still difficult, since some of these immortalized cells had converted to malignant growth. It seems to be relevant, however, that even malignant HPV-positive cells can be converted to senescence by the introduction of chromosome 11 [246–249] chromosome 4 [250] chromosome 2 [251] and chromosome 1 [252]. Unfortunately, most of these studies, particularly those on chromosome 11, and 2, were performed with malignant lines, rendering an interpretation of the relationship of these suppressing events strictly to immortalization impossible.

Cellular genes engaged in the prevention of virus-induced immortalization have not yet been identified. It has been speculated that one of these genes may code for the cyclin-dependent kinase inhibitor $p16^{INK4}$ protein [253]. This protein is upregulated under conditions of pRB inactivation [254–256]. Since the pE7/pRB interaction results in pRB inactivation (see Section 4.5 on E7), the upregula-

tion of $p16^{INK4}$ may account for the growth limitation of primary HPV-infected cells resulting in senescence after a prolonged lifespan. This could provide an explanation for the functional impairment of senescent HPV oncoprotein-expressing cells. In spontaneous immortalization of Li-Fraumeni syndrome fibroblasts loss of $p16^{INK4}$ expression has been consistently observed [257]. A very recent study suggests that loss of $p16^{INK4}$ may be required for the immortalization of human uroepithelial cells by HPV 16 E6, but not for those immortalized by E7 or jointly by E6/E7 [258]. More studies need to be done on $p16^{INK4}$ expression in HPV-immortalized cells to clarify the role of this gene product and of other cyclin-dependent kinase inhibitors in the suppression of immortalization.

It is unlikely that p53 is directly involved in the control of immortalization. This can be deduced from experiments in mice where a p53 knock-out phenotype results into a relatively normal development of these animals with an increased tumor incidence in later life [259]. It is however highly likely that p53 plays a very important indirect role for the progression of HPV infected cells towards immortalization and even to a malignant phenotype [208]: the inactivation of p53 by the E6 protein emerges as the responsible event for the prevention of the p53-mediated G1 arrest following DNA damage [224,136]. Accumulation of resulting mutations in the course of subsequent cell divisions is probably a most important precondition for the eventual selection of cell clones which acquired mutations in cellular genes controlling immortalization. *The functionally inactive p53 thus seems to represent the most important progression factor*, possibly without a direct role in immortalization and transformation.

It has been speculated that the stability of telomere sequences regulated by the telomere polymerase plays an important role for cell proliferation and senescence [260]. Indeed, telomere shortening is consistently observed under conditions of cellular senescence [378], whereas activation of telomerase and recovery of telomere length with telomere stabilization occurs in immortalized cells [261]. Telomere shortening is also observed in normal human and pre-crisis HPV-expressing cells with a recovery of telomere length after immortalization [391]. A recent study showed that telomerase becomes activated by the E6 gene product of human papillomavirus type 16 even in keratinocytes which do not become immortal after E6 introduction [262], indicating that telomerase activation is insufficient for immortalization.

Hormones may play an important role in in vivo events related to immortalization (see below) of HPV infected cells. High risk HPVs harbor glucocorticoid responsive elements within the long control region [263]. Glucocorticoids enhance substantially immortalization by HPV 16, but fail to induce the same activity in HPV 11 infections [264,265]. The hormone-dependent transformation by HPV 16 and ras can be inhibited by a hormone antagonist RU 486 [266]. Although all these studies were performed under tissue culture condition, it is suggestive that reported marginal increases in the risk for cervical cancer development under long-time oral contraceptive may be related to these observations [267,268].

9. Malignant progression

Numerous studies indicate that there exists a substantial time lag between primary infection by high risk HPVs, development of cervical intraepithelial neoplasias, carcinomata in situ, and finally invasive cancer. Anogenital HPV infection occurs most frequently at young age with the onset of sexual activity and depending on the number of sexual partners. High rates of HPV detection have been reported in females in Western countries in age groups between 16 and 20 years [193], summarized in [388]. Cervical intraepithelial neoplasias reveal a peak incidence in age groups between 25 and 35 years, whereas cervical cancer incidence peaks between 55 and 65 years of age [191]. This suggests already that the latency period between primary infection and development of intraepithelial neoplasia averages several years, that the progression towards invasive growth seems to require additional 20 to 30 years [207].

The molecular basis for this long time span becomes increasingly understood: in situ hybridization studies [79,98] and analyses of HPV16 E6/E7 transgenic mice [269] indicate an incremental upregulation of HPV E6/E7 expression at each stage of neoplasia. Inversely, downregulation of E6/E7 transcription takes place in HPV-immortalized non-malignant cells after heterografting them into immuno-compromised animals [270,271] or by exposing them to human macrophages in vitro [272]. This results at the same time in a marked growth inhibition. Finally, the selective inhibition of HPV18 E6/E7 gene expression in cervical carcinomas cells results in growth inhibition and loss of tumorigenicity [220,49]. All these data show that high risk HPV E6/E7 expression is a prerequisite for continued growth stimulation. Its upregulation in the course of progression suggests a correlation between the quantity of the viral oncogene product and the severity of the lesion. The selective E6/E7 downregulation after heterografting non-malignant cells into immuno-compromised animals shows, moreover, that the regulation of viral oncogene expression differs between immortalized and malignant HPV-harboring cells.

10. The CIF-concept

In Section 8, the role of the failure of a controlling signalling cascade *interfering with the function* of high risk HPV oncoproteins for the development of the immortalized phenotype has been discussed. Thus, immortalization requires viral oncogene expression, but, in addition

the functional failure of alleles regulating this signalling cascade. The reasons for suspecting a proportion of low grade cervical intraepithelial neoplasias as correlate of a similar in vivo failure were also summarized there. Malignant progression obviously depends on additional changes within the genome of the infected cell. As pointed out in the preceding paragraph, apparently an additional signalling cascade needs to be interrupted, *interfering with the transcriptional activity* of the viral oncogenes. The existence of a cellular interference factor (CIF) was initially postulated in 1977 [273,207,406]. The emerging picture today points to the existence of *two CIF-cascades*, whose sequential interruption in an HPV-positive cell is the precondition for HPV-linked cancers.

Early evidence for the existence of a cellular control of viral oncogene transcriptional regulation originated from studies revealing a selective downregulation of HPV transcription in non-malignant HeLa-fibroblast hybrids by 5-azacytidine treatment in contrast to parental HeLa cells [274]. Similarly, the HPV promoter, regulating the transcription of HPV oncogenes in cervical carcinoma cells, was silenced after subjecting these cells to somatic cell hybridization with normal keratinocytes [275]. The downregulation of E6/E7 transcription in non-malignant cell hybrids and immortalized cells after heterografting them into susceptible animals [270,79,98] further supported this interpretation. These experiments pointed to a paracrine regulation of viral oncogene transcription. The demonstration of an HPV transcriptional repressing effect of human and murine macrophages [272] and unpublished) and of specific cytokines excreted by activated macrophages, like TNFα and Interleukin-1 [276,394,272] and TGFβ [277], suggests a cytokine-mediated response to paracrine signals.

Individual steps of this signalling cascade still have to be elucidated. Besides specific cytokine receptors, it is likely that a region in the short arm of chromosome 11, apparently engaged in the regulation of protein phosphatase 2A (PP2A), plays an important role: deletions in this region lead to an upregulation of a regulatory subunit of PP2A, enhancing HPV transcription in human fibroblasts [278]. Similar effects have been noted after inhibition of the catalytic subunit of PP2A by ocadaic acid or SV40 small t antigen.

The terminal effect of this signalling cascade should be mediated by specific transcription factors. A number of these factors have been shown to be important for the regulation of HPV transcription (reviewed in [53]. A number of negative regulators have been identified: the Oct-1 transcription factor [53], the nuclear receptor for Il-6, NF-Il-6 [276], retinoic acid receptors [279–281] and YY-1 [56]. Except for Oct-1, the other factors show some differential activity in non-malignant when compared to malignant cells. YY-1 interacts with an upstream 'switch'-region in HPV promoter suppression [282].

Among factors positively regulating the HPV promoter (see review [53]), AP-1 seems to play a particularly important role [283,284,52]. Modification of the proximal AP-1 binding sites results in a loss of transcriptional activity. Recent data indicate that in the course of antioxidant or TNFα-induced transcriptional inhibition of E6/E7 transcription the composition of AP-1 complexes changes [401]. C-*jun* and *jun-B* heterodimerize with *fra-1*, the synthesis of these proteins is substantially enhanced in non-malignant cells under such conditions. In spite of an increased synthesis as well of *c-fos*, this protein is no longer found in AP-1 complexes and may be rapidly degraded. Thus, the upregulation of *fra-1*, probably triggered by cytokine interaction, and its increased presence in AP-1 heterodimers could represent one of the decisive factors in the differential control of HPV transcription in non-malignant cells. *Fra-1* is not upregulated in the malignant cervical carcinoma cells tested thus far.

The disruption of cellular signalling cascades resulting from modifications of host cell DNA sequences requires mutational events or epigenetic modifications (e.g., methylation) of cellular DNA. In high risk HPV infections this is most likely mediated by mutagenic activity of the E6 and to a lesser degree the E7 oncogene expression (see Section 4.5.1 and Section 4.5.2). Continued expression of these oncoproteins, besides their inherent growth-stimulatory activity, will gradually lead to a selection of cell clones with specific mutations, permitting further dysregulation of viral oncogene expression and viral oncogene activity. A schematic representation of these events is outlined in Fig. 2. Thus, high risk HPVs seem to act as solitary carcinogens.

11. Hereditary factors in papillomavirus susceptibility and carcinogenesis

Persistence and appearance of papillomavirus-induced lesions is obviously influenced by genetic factors, at least in a number of conditions: The genetic predisposition for epidermodyslasia verruciformis has been discussed before. Similar data exist for focal epithelial hyperplasia of the oral mucosa [285–287] which occurs at high frequency among American Indians and in Eskimos.

For squamous cell carcinomas of the cervix a high risk has been reported in women with HLA DQw3 [288]. Although these data have been disputed in additional publications [289,290], they are supported by similar observations in rabbit systems [291].

It appears to be very likely that our increasing knowledge of mutations of specific cellular genes, controlling HPV oncoprotein function or viral oncogene expression, will lead to the identification of a higher number of individual genes whose failure will predispose to cancer development. The existence of these modified genes within the germ line will depend on the compatibility of these modifications with the development and survival of the affected embryo.

12. Role of viral DNA integration

It has been noted early after the identification of HPV DNA in cervical cancer that the viral DNA regularly becomes integrated into host cell DNA [32,76,292,398]. The integrational events results in an interruption of the E2, frequently also of the E1 open reading frames [76] and to deletion or partial deletion of the downstream genes E4, E5, and L2. The E2 protein codes for transcriptional regulatory proteins that can both activate and suppress viral transcription [73,293]. It has been shown that the disruption of this intragenomic regulation leads to dysregulation of viral E6/E7 transcription and increases the immortalization capacity of the HPV16 genome [77]. The integration also leads to an increased stabilization of the viral message, apparently by inactivating a destabilizing element in the 3'-region of the viral mRNA. This stabilization is resulting from the chimaeric composition of E6/E7 message with flanking host cell sequences [76].

Viral DNA integration and dysregulation of E6/E7 oncogene activity is clearly not sufficient for the development of a malignant phenotype. Integration of viral DNA has been noted in a number of high grade intraepithelial lesions [294,295]. Moreover, somatic cell hybridization of cervical carcinoma cells with normal cells regularly leads to a non-malignant phenotype, inspite of the continued presence and expression of integrated viral DNA [270]. Finally, in up to one third of cervical biopsies exclusively episomal persistence of viral DNA has been noted [78].

Integration adds, however, to the dysregulation of viral oncogenes. The most likely mode of this dysregulation is probably less an increase in transcriptional activity rather than the increased stability of E6 and E7 mRNA as a result of the disruption in the 3' untranslated part of the early viral region. The A + U-rich element within this region confers instability on a heterologous mRNA [296]. Indeed, a higher level of E7 protein has been found in clonal cell populations of human cervical epithelial cells containing integrated HPV 16 DNA when compared to those containing exclusively extrachromosomal viral DNA [297].

13. Specific chromosomal aberrations in cervical cancer

The prediction of interrupted cellular signalling cascades in the development of cervical cancer should stimulate studies to identify specific chromosomal aberrations in these malignant, but also in premalignant lesions. Nonrandom structural changes and numerical chromosome aberrations have been reported in cervical cancer [298–300]. Loss of heterozygosity (LOH) was most frequently observed in chromosome arms 6p21-23, 3p13-25, and 18q12-21. A number of other loci with LOH were identified on 16 additional chromosome arms, with higher frequency for chromosomes 11p and 11q and chromosome 17p [300]. A rearrangement of chromosome 11q13 has been observed in three cervical carcinoma cell lines [301]. It may be of substantial interest that this chromosomal region also harbors the fra-1 gene [302]. A gene (HTS1) in chromosome 11p15 was localized and identified as a tumor suppressor gene for HeLa cells [303].

Integration of viral DNA may also occur into specific chromosomal loci potentially engaged in the control of viral oncogenes resulting in the selection of such clones ([304] and unpublished data). The region 10q24 has been identified in these studies with LOH in several cervical carcinoma biopsies. Another integration site has been found in chromosome 12q14-15 in two cell lines (SW756 and SK-v) derived from genital tumors [413].

Specific genes modified in some cervical cancer biopsies involve the jun-B gene [305], the ETS2 oncogene [248] and the DCC gene [306]. Their role in HPV oncogene regulation remains to be established.

14. Papillomaviruses in human cancers

14.1. Papillomaviruses in cancer of the cervix and in other anogenital cancers

HPV 16, 18, and a number of additional HPV types have been found in about 95% of all biopsies derived from cancer of the cervix throughout the world, when testing was performed with a sensitive technology and screening permitted the detection of more than 4 HPV types [307,308,34]. HPV16 is by far the most frequently found virus type and accounts for 50 to 60% of all positive data, the presence of HPV18 varies between 10 and 20%. A number of analyses failed to include specific testing for HPV45, a type relatively closely related to HPV18. Resulting cross-hybridizations may have led to an overestimation of HPV18 positivity. Many of these types have been found in exceptional cases only.

The mere presence of HPV DNA in the vast majority of biopsies from cancer of the cervix does not prove an etiological involvement (see above). Early data pointing to a role were derived from experimental findings which have been discussed in the previous sections:

- the regular expression of HPV E6/E7 genes within the cancer cells,
- the absence of detectable cellular regulation of E6/E7 genes in cancer cells,
- the immortalization of human cells by the expression of these genes,
- E6/E7 protein induction of growth promotion and chromosomal instability,
- cessation of cell growth and reversion of the malignant phenotype in cells of cervical carcinoma cell lines after selective blocking of E6/E7 gene function.

These data essentially demonstrate that the E6/E7 oncoproteins are the main determinants of the malignant phenotype in those cervical carcinoma cells which have

been tested thus far. They provided the background for epidemiological studies analyzing the role of HPV in cancer of the cervix.

Case-control studies contributed mainly to the epidemiological link of HPV infections to cancer of the cervix. Although initial studies suffered from problems related to test validity and study design [309,310], later studies were better controlled for known or suspected risk factors for cervical cancer (summarized in [388]). In a careful population-based case-control study [32] analyzed 436 incident cases of squamous cell invasive cancer and 387 population controls in a low risk country (Spain) and a high risk country (Colombia). The odd ratios (OR) for HPV DNA were 46.2 in Spain and 15.6 in Colombia. Among HPV-negative cases, the risk factors identified were related to sexual behaviour. In the HPV-positive group the only other recognizable risk factor (besides HPV) were the use and duration of oral contraceptives intake [311]. The majority of 28 other studies came up with OR values between 10 and 50, five of these were below 5, five of them above 50 [388]. Two of the latter exceeded OR values of 100. By including only those studies which used PCR technology, the OR slightly exceeded 50 in average. Thus, the OR for anogenital HPV infections as risk factors for cancer of the cervix clearly and substantially tops those reported for long-time tobacco smoking and lung cancer [387].

Cohort studies have been performed to study the transition of HPV infection to cervical intraepithelial neoplasia (CIN) and the progression from CIN to cancer. They have been summarized previously [388]. A careful analysis by [312] revealed a relative risk (RR) of HPV-positive women for the development of high grade CIN of 11. All other available prospective data indicate that HPV16 and 18 infections precede high grade CINs and predict an elevated risk to develop these lesions (see [388]). Thus, epidemiological studies now strongly support the experimental data implicating specific HPV types as cervical carcinogens. The absence of significant other identifiable risk factors also supports the experimentally derived concept [208,386,399] that high risk HPV types may act as solitary carcinogens.

The combined sets of experimental and epidemiological data prove the etiological role of specific HPV types in a high proportion of cervical cancers. The presence of additional types in cervical carcinoma biopsies renders it likely that they also play a decisive role in the development of these cancers. It is presently an open question whether the remaining approximately 5% of 'virus-free' cervical cancers are indeed not harboring viral DNA, and thus may have been caused by other factors, or whether they contain yet not identified novel HPV types. Although it is likely that the majority of anogenital HPV infections has been identified by now, there still exists the possibility of additional infections.

In cancer of the vulva a number of studies reported a lower incidence of HPV positivity when compared to cervical cancer [388]. Sensitive studies which included testing for more types than HPVs 6, 11, 16, and 18 reported positive findings between 30 and 86% [313–315], with an overall positivity slightly above 50%. There appear to exist two different types of vulvar cancer: squamous cell carcinomas affecting a younger age group, preceded by vulvar intraepithelial neoplasia, containing HPV DNA (most frequently HPV16); and another group occurring at higher age with lichen sclerosus-like lesions devoid of detectable HPV DNA [316,317]. It will be interesting to analyze tumors of the latter group for those cutaneous HPV types which have been recently found in squamous cell carcinomas of the skin in immunocompromised and immunocompetent patients [198].

The number of vaginal cancers tested for HPV DNA is still small. 52 cases have been reported in the literature, tested under conditions of different sensitivity and specificity. 35 of these biopsies were tested by procedures which permitted HPV typing beyond the four standard types. Twenty of these (57%) turned out to be HPV-positive [318,319,389]. Since the number of types analyzed in these tests was still very limited, this percentage of positivity most likely represents an underestimate of the real figure.

In penile cancer, the situation appears to resemble that of vulvar cancer, although the overall rate of HPV positivity seems to exceed that of the latter [388]. Studies which included a larger spectrum of types ranged in their positivity between 54 and 100% [320–323] with an average of 73% positivity. Most frequently again HPV16 is detected in these tumors. The total number of tumors tested under sensitive and specific conditions is here very limited, too. Yet, there may also exist an entity of penile cancers without the DNA of anogenital HPV types.

The rare verrucous carcinomas of penis or vulva and Buschke-Löwenstein tumors frequently contain HPV6 or 11 DNA [324].

In anal and perianal cancer the limited number of available studies describe a high degree of HPV positivity, exceeding 70%, when testing was performed under appropriate conditions [325,326]. Here the situation seems to resemble cancer of the cervix, although the limited number of tests does not permit firm conclusions. Again, the small number of HPV types analyzed in these biopsies renders it highly likely that the available detection rates represent underestimates.

In summary, papillomavirus DNA is detectable in at least 95% of cancers of the cervix and in more than 50% of vulvar, vaginal, penile, anal, and perianal cancers.

14.2. Papillomaviruses in non-melanoma skin cancers

Early studies on papillomaviruses in skin cancer were conducted in patients with epidermodysplasia verruciformis (EV) (reviewed in [188]. EV is a rare hereditary condition occurring worldwide, in which initially benign

papillomatous lesions arise frequently at the age of 5 to 8 years. Some of these lesions appear as red plaques and are often overlooked by the patients. In about one half of the patients the lesions progress within the following 20 to 30 years. At light-exposed sites initially actinic keratoses and Bowenoid changes develop. Cancer progression occurs mainly on the forehead and on traumatized sites, resulting in local destruction, usually without metastazation.

More than 20 individual types of HPV have been demonstrated in benign lesions of EV-patients. The same patient frequently reveals several HPV types in different lesions. Interestingly, squamous cell carcinomas developing in these patients usually contain HPV5, some of them HPV8, but rarely other HPV types [327].

Although specific HPV types have been found in squamous cell carcinomas of EV patients already in 1978, their causal role for the induction of these cancers, though suggestive, is not proven in a formal sense. This is due to the difficulties in obtaining EV HPV-positive cell lines and to the inability of these viruses to immortalize human cells. In view of the observations that most of the malignant lesions develop at light-exposed sites, it is likely that an interaction between sunlight exposure and HPV infection represents the major reason for cancer development. The exact nature of this interaction remains to be established.

The underlying genetic effect of predispositions to epidermodysplasia verruciformis is not yet clear. Interestingly, these patients do not show an increased sensitivity to anogenital HPV infections. It has been described that EV patients reveal an inhibition of natural killer cell activity [328] and of cytotoxic T cells [329]. As a constant abnormality, cutaneous anergy to strong contact sensitizers (dinitrochlorobenzene - DNCB) has been noted in EV patients [330]. EV-like lesions have also been observed in immunosuppressed patients following organ transplantation [331–334] or after infection with human immunodeficiency virus [335,336].

Early studies on non-melanoma skin cancers were mainly conducted by using probes specific for anogenital HPV types [337,338]. With the exception of periungual Bowen's disease and periungual squamous cell carcinomas [339,177,340–342] which regularly contain HPV 16 DNA, a low percentage of HPV positivity was noted (reviewed in [388]). One notable exception was the finding of HPV 41-positive cancers in 2 out of 10 squamous cell carcinomas [343].

Recently, the use of consensus primers covering a broad spectrum of HPV types changed the picture substantially: EV types [344] but also a larger number of novel HPV types [198] were discovered in about 80% of squamous and basal cell carcinomas of immunosuppressed patients. Even in immunocompetent patients, approximately 30% of these tumors contained identifiable DNA, when tested with 16 different combinations of consensus primers [198]. A large number of different HPV types have been found in these tumors without a prevalence of specific types. This is a rather puzzling result and presently difficult to interpret. Since these types of tumors again develop almost exclusively at sun-exposed sites, an interaction between these virus infections and solar UV-irradiation could represent an explanation. Probably all these virus types represent low risk infections and are non-mutagenic for host cell DNA. Only additional mutations induced by sun exposure may result in malignant progression. This, of course, requires further investigation.

The present unavailability of suitable in vitro systems to analyze the biological activity of cutaneous HPV infections contributes to the difficulties in assessing their role in skin tumor progression.

There exist no reports on extensive testing of melanomas for HPV DNA. One positive finding of HPV37 in one melanoma remained solitary up to today, the same study failed to find this DNA in 35 additional melanomas or in 190 other skin tumors [345].

14.3. Papillomaviruses in cancers of the oral cavity, the larynx, the lung and the nasal sinuses

Cancers of the oral cavity, the tongue, the hypopharynx, and the larynx have been mainly analyzed for anogenital and cutaneous HPV infections. The vast majority of identified HPV types originates from these two sites, only very few specific types have been isolated from the oral cavity [202,226,201]. In view of the frequent occurrence of papillomas or papilloma-like lesions in the oral cavity [346], reviewed in [347], among them a fair percentage without HPV DNA detectable by the probes used in these studies, it is likely that careful screening will lead to the discovery of additional papillomavirus types, specifically infecting these mucosal sites.

The first reports on the presence of HPV in malignant tumors of the oral cavity appeared in 1985 [348,349]. They were followed by similar reports on HPV DNA in individual cases of cancer of the larynx [350–352]. Although subsequently, by using PCR analysis, the HPV detection data varied between 0 and 100% (see [388]), studies performed with reliable techniques commonly came up with positivity rates between 10 and 20%. In these studies most frequently HPV16 has been found, HPV18, HPV6, HPV11, and HPV2 were also reported in individual biopsies. One specific form of oral cancer, tonsillar carcinomas, revealed DNA of anogenital HPV types at exceptional frequency, exceeding 50% of the biopsies tested [353–355].

In laryngeal carcinomas the situation resembles that of other oral cancers. Again a wide variety of data has been published ranging between 0 and 100% (see [388]). It appears, however, that the situation is not different from other oral cancers, in that an average of close to 20% is presently positive when tested by reliable procedures. Again most reported data preferentially included anogenital types as probes.

Patients with laryngeal papillomatosis with subsequent development of laryngeal or lung cancer contained HPV6 or 11, and in one case HPV16 [356–359]. Other lung carcinomas were only exceptionally reported to be positive for HPV DNA [360,361]. Other studies failed to detect HPV DNA in this type of cancer [362,363]. By using a wide spectrum of consensus primers, [364] failed to find positive tumors in a larger series of these cancers. Thus, lung cancer with few exceptions seems to be devoid of presently detectable HPV genomes.

In carcinomas of the nasal sinuses, HPV 16 and 18 have been recorded (see [388]), in addition to HPV 57 which is more frequently found in inverted papillomas [365–367].

14.4. Papillomaviruses in cancers of the esophagus

A role of papillomavirus infections in cancer of the esophagus has been postulated initially by Syrjänen in 1982 [403], based on histological changes similar to those of condylomatous lesions in esophageal squamous cell carcinomas. Inspite of numerous attempts to clarify the role of HPV in this cancer (see [388]), the available data do not permit firm conclusions. The available studies are frequently based on in situ hybridizations or solely on PCR technology. A Southern blot hybridization study performed by [368] failed to provide evidence for positive tumors, although the same authors find close to 50% positivity by applying a PCR reaction specific for anogenital HPV types. In view of additional PCR data reporting positive results in the range between 7 and 24% [369–371], it is likely that a certain percentage of these tumors contains identifiable types of HPV genomes. A careful reappraisal of these data by using tests with a broad reactivity is clearly desired in order to elucidate the role of HPV in this frequent form of human cancer.

14.5. Additional cancers suspected to be linked to papillomavirus infections

Although a number of reports have been published claiming the presence of HPV DNA in human prostatic cancer, in cancer of the ovary, the colon, and cancer of the breast (see [388]), these studies have not been confirmed in other laboratories and remain at present unexplained.

Well-documented individual cases, however, exist in cancers of the bladder [372–374] and urethra [375,376,404]. The percentage of positive tumors in bladder cancer is usually low; in most studies below 10%. In urethral cancers higher rates of positivity have been noted. This is not unexpected since the latter site may be directly exposed to HPV infections.

15. The global role of HPV-linked cancers and conclusions

Cervical cancer represents the second most frequent malignant tumor of women worldwide with an estimated frequency of approx. 440000 new cases per year, corresponding to about 5.8% of the global cancer incidence [400]. If one considers that more than 50% of vulvar, penile, perianal and anal cancers, in all likelihood more than 20% of oral, laryngeal and nasal cancers contain predominantly anogenital high risk HPV types, this leads to almost 10% of the worldwide cancer burden linked to these infections.

Recent data point also to a role of low risk types in non-melanoma skin cancer, here in conjunction with a physical carcinogen, solar exposure. Non-melanoma skin cancer is the most frequent malignancy in caucasian populations. Although the latter data need to be substantiated, there is good reason to suspect that the actual contribution of papillomavirus infections to human cancers well exceeds the 10% mark. This identifies infections by certain members of this virus group as one of the most important risk factor for human cancer development. In view of rather excellent prospects of controlling at least some of these infections by vaccination [377], the identification of an HPV etiology of a major fraction of human cancers paves the way for new strategies in cancer prevention.

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A Molecular Portrait of Human Papillomavirus Carcinogenesis

T.R. Broker,* L.T. Chow,* M.T. Chin,* C.R. Rhodes,*
S.M. Wolinsky,†§ A.Whitbeck,‡ and M.H. Stoler‡

*Biochemistry Department, Cancer Center; †Department of Medicine, Infectious Diseases Unit; ‡Pathology Department, Cancer Center; University of Rochester School of Medicine, Rochester, New York 14642

The human papillomaviruses (HPVs) specify a complex family of overlapping polycistronic mRNAs, including one that encodes transformation proteins and an autoregulatory transcriptional factor capable of both positive and negative feedback. The viral enhancer-promoter region also responds to constitutive and conditional host transcriptional factors. Together these regulatory proteins modulate viral transcription in coordination with epithelial tissue differentiation. We have designed probes that can distinguish each of the five prevalent and related HPVs that infect the anogenital region. We have also built subgenomic clones from which riboprobes specific for individual mRNA species are generated in vitro. These probes reveal the viral transcription patterns in serial sections of patient biopsies after in situ hybridization. Our studies on a spectrum of lesions ranging from benign epithelial hyperproliferation to invasive carcinomas associated with HPV types 6, 11, 16, and 18 demonstrate that viral gene expression is tightly linked to cellular differentiation. The most definitive parameter in characterizing the state of viral-host interactions associated with the oncogenic HPV types 16 and 18 resides in the relative abundances of mRNAs from the E6-E7, E4-E5, and L1 regions. In low-grade lesions, all are expressed, with the E4-E5 RNA being most abundant. As the severity of neoplasia increases, the expression of E4-E5 and L1 decreases or becomes absent. Conversely, the E6-E7 region is derepressed. On the basis of this information, we propose a molecular mechanism for the derepression of the viral transformation genes. The recently described interactions between the viral transformation protein and the retinoblastoma (RB) anti-oncoprotein and perhaps others probably also play key roles in the initiation of viral carcinogenesis (Dyson et al. 1989 and this volume).

Neoplasia of the uterine cervix has traditionally been classified according to histopathologic criteria (Patten 1978; Koss 1979). Intraepithelial neoplasms appear to form a morphologic continuum, with clear evidence for progression through successively more severe stages to invasive cancer. Virtually all of these morphologic alterations have been consistently associated with one or more types of HPV infection. Nearly two dozen HPV types have been found in the anogenital tract. HPV types 6 and 11 are most often associated with benign venereal warts (condylomata acuminata) in the lower genital tract and are also infrequently found in some carcinomas. In contrast, higher-grade intraepithelial neoplasia, carcinomas in situ, and invasive carcinomas are associated primarily with HPV types 16, 18, 31, 33, and 35 (for review, see Pfister 1987). This paper describes molecular studies of HPV transcription and regulation, relates them to the transcription profiles observed in a spectrum of HPV-16- and HPV-18-associated genital tract lesions and proposes a molecular mechanism for HPV-induced carcinogenesis.

§Present address: Northwestern University School of Medicine, Department of Medicine, Infectious Diseases Unit, Chicago, Illinois 60611.

The normal epithelium of the uterine cervix varies according to anatomic site. The lining of the endocervical canal is a mucus-secreting, unstratified columnar epithelium. The ectocervix is covered by a stratified noncornified squamous epithelium. After puberty, the position of the squamo-columnar junction generally moves up the endocervical canal as glandular epithelium becomes replaced by metaplastic squamous epithelium. This transformation zone, where active epithelial turnover is most evident, is also the site for initiation of most of the epithelial neoplasms of the cervix. All of the epithelial cell types are probably derived from a common pool of dividing multipotential reserve cells that can differentiate along a variety of paths, depending on regional stimuli (Coppleson and Reid 1967; Gould et al. 1979). In squamous mucosae, the dividing cell population is generally restricted to the basal and possibly parabasal layers. A subpopulation of daughter cells are pushed up toward the surface. These cells undergo several successive changes in the expression of particular subsets of the keratin gene family (Moll et al. 1983). Such differentiation is reflected in the characteristic cellular morphology in a given epithelial cell layer. Under normal conditions in the ectocervix, the mature squamous mucosa does not have a well-defined granular or keratin

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layer. The superficial squamous cells contain abundant cytoplasm and a small pyknotic nucleus and are the primary cells sloughed to the environment. In contrast, external cutaneous skin matures several steps further to produce a highly cornified surface layer.

Papillomaviruses induce changes in differentiating epithelia. The most pathognomonic cell, often seen in productive infections, is called a koilocyte (koilo means cave), which has a large vacuole around the nucleus. The nuclei of koilocytes are almost always atypical in that they are enlarged and hyperchromatic, with an associated increase in nuclear to cytoplasmic ratio. HPVs also induce architectural changes in the epithelium, including papillary hyperproliferation and epithelial thickening (acanthosis), thought to result from the enlargement of individual cells and from a delayed course of differentiation leading to the accumulation of excessive numbers of cells prior to desquamation (Steinberg 1986). These features are characteristic of the usually benign exophytic neoplasm, condyloma acuminatum of the vulva and vagina. However, in the cervix similar changes are more frequently associated with flat epithelial proliferations lacking papillary growth. Such "flat condylomas" are recognized as the earliest manifestation of a spectrum of cervical dysplasia (intraepithelial neoplasia). Morphologic progression through the stages of dysplasia includes replacement of increasing proportions of the epithelium above the parabasal stratum with dividing cells that often exhibit nuclear abnormalities, such as increased numbers of mitoses, abnormal mitoses (aneuploidy), and multinucleated cells. There is concurrently a proportional decrease in the frequency of koilocytes, presumably because of the absence of the differentiation signals necessary for the production of viral cytopathic effects associated with late viral gene transcription. Dysplasias are graded as mild (cervical intraepithelial neoplasia grade I [CIN I]), moderate (CIN II), or severe (CIN III) corresponding, respectively, to the replacement of one-third, less than two-thirds, or greater than two-thirds of the epithelium by basal-like cells. Classic carcinoma in situ of the cervix (also called CIN III) is recognized as a stage in which the entire epithelial thickness is replaced by primitive undifferentiated cells.

Squamous carcinomas of the cervix generally arise from, or in association with, squamous intraepithelial neoplasms. They may have keratinizing, nonkeratinizing, or undifferentiated morphology, and may be composed of large or small cells exhibiting a range of individual cell differentiation. Adenocarcinomas of the cervix arise in glandular epithelium, often associated with a morphologic precursor, in situ adenocarcinoma. Small-cell undifferentiated carcinomas do not have a well-defined histogenetic precursor and frequently demonstrate neuroendocrine differentiation. They are analogous to small-cell carcinomas of the lung and are characterized by a particularly aggressive clinical course. The developmental relationships among these three types of epithelial differentiation are not certain, but it appears that the reserve cell population is capable of differentiating along all three directions. Initiation of neoplastic development could conceivably occur in a cell already committed to one of the differentiation paths. Alternatively, carcinogenesis may be initiated in a common multipotent precursor cell, with the subsequent differentiation influenced by genetic and environmental events during tumor progression. Because tumors with mixed differentiation are not unusual, a common stem cell capable of multipotential differentiation is quite possible. We present some additional evidence based on patterns of HPV gene expression in favor of a common origin of these tumors. Similar patterns of histopathogenesis are recapitulated in other anatomical sites, including the male urogenital tract, the anal mucosa, and the nasal, laryngeal and respiratory mucosa, all of which have morphologically similar transformation zones and are subject to HPV infection.

Papillomavirus genome structure, transcription, and regulation

Numerous human and animal papillomavirus genomes have been cloned, and for some, their DNA sequences have been determined. They are a family of related double-stranded DNA viruses with circular genomes of approximately 7900 bp that replicate as extrachromosomal plasmids in the nuclei of epithelial cells in benign lesions. Their genomes are all similarly organized into early and late open reading frames (ORFs) encoded by the same DNA strand (Fig. 1C). Immediately preceding the early ORFs is an upstream regulatory region (URR) or a long control region, which contains transcriptional enhancer elements, promoters, and DNA replication control sequences (for review, see Broker and Botchan 1986). The early (E) region encodes *trans*-acting factors required for regulated, extrachromosomal replication (E1 ORF), enhancer activation and repression (E2 ORF), and cellular transformation (E5, E6, and E7 ORFs). The viral capsid proteins (L1 and L2) are the products of the late region and are expressed in productive lesions but not in transformed cells.

In benign HPV-infected lesions, the viral DNAs exist as extrachromosomal plasmids, mostly as monomeric circular molecules. However, in some but not all cancers associated with HPV-16, viral DNAs are found as multimeric circular molecules, sometimes with deletions (Dürst et al. 1985; Choo et al. 1987; Smotkin and Wettstein 1987). In other cancers, viral DNA is integrated into host chromosomes (Dürst et al. 1985, 1987; Matsukura et al. 1986; Shirasawa et al. 1986; Choo et al. 1987). Viral integration, when characterized, invariably disrupts the E2 ORF encoding the transcription regulatory proteins (Schwarz et al. 1985; Matsukura et al. 1986; El Awady et al. 1987; Baker et al. 1987; Choo et al. 1988). Naturally occurring malignant lesions express viral E6 and E7 mRNAs (Smotkin and Wettstein 1986; this paper). In addition, most long-established cervical carcinoma cell lines, such as HeLa, SiHa, and CaSki, have been found to harbor integrated HPV types 16 or 18 DNA from which the transforming E6 and E7 regions are actively transcribed (Pater and Pater 1985;

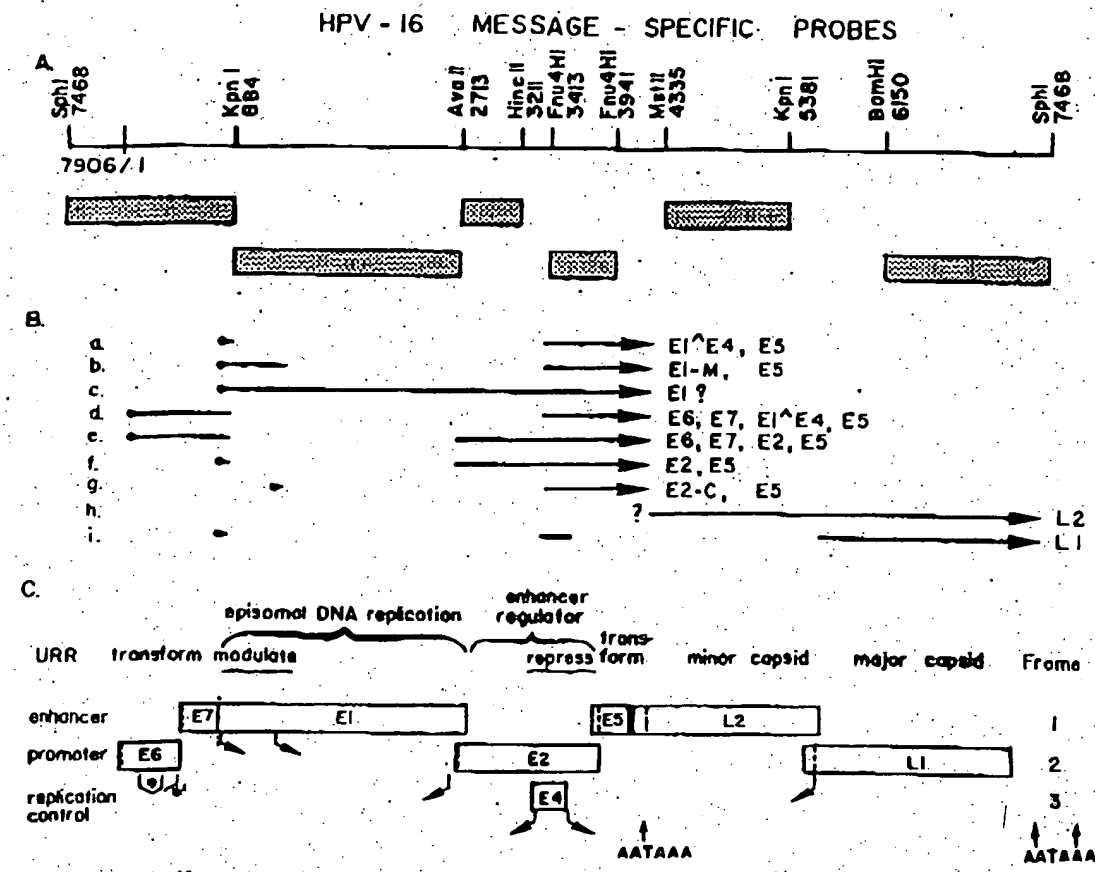

Figure 1 HPV-16 exon-specific probes. (*A*) Subgenomic segments of HPV-16 DNA corresponding to the anticipated mRNA exons were cloned into pGEM dual promoter vectors. (*B*) A representative set of HPV-6 and HPV-11 mRNAs (Chow et al. 1987a) and their probable protein-coding potential. (*C*) The HPV-16 ORFs based on DNA sequence (Seedorf et al. 1985) and the probable functions of the encoded proteins. RNA splice donor and acceptor sites when known or inferred from homology with HPV-11 are marked with bent arrows.

Schwarz et al. 1985; Yee et al. 1985; Schneider-Gädicke and Schwarz 1986; Baker et al. 1987). These observations further strengthen the important relationship between these HPV types and cervical neoplasia.

Cellular transformation in vitro also has pointed to an active role for certain papillomavirus types in these processes. HPV-16 DNA immortalizes cultured primary foreskin keratinocytes or primary cervical cells in culture (Pirisi et al. 1987; Woodworth et al. 1988). HPV types 16, 18, 31, and 33, but not HPV types 6 or 11, are capable of transforming primary baby rat kidney epithelial cells in collaboration with an activated cellular oncogene, Ha-*ras* (Storey et al. 1988), thus mimicking the multistep carcinogenesis experiments described by Land et al. (1983). HPV-16 also inhibits the ability of primary keratinocytes to differentiate when cultured on collagen rafts at the air-media interface (Asselineau and Prunieras 1984; Kopan et al. 1987), inducing morphologic transformation that mimics CIN (McCance et al. 1988). In such experimental systems, the transformed phenotype is not apparent until the cells have been passaged many generations, again suggesting the need for additional genetic events. The major transformation gene of HPV-16 is E7 (Phelps et al. 1988; Storey et al. 1988). The E7 protein has been identified in cervical carcinoma cell lines (Smotkin and Wettstein 1986) and in transformed rodent cells (Banks and Crawford 1988). Recently, a homology between the HPV E7 protein and other viral and cellular oncogenes has been recognized (Phelps et al. 1988). Moreover, these proteins form complexes with the recently characterized RB anti-oncoprotein (Lee et al. 1987; DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989 and this volume), suggesting the interaction between E7 and RB proteins could play a role in HPV-induced maligant transformation. It is not known whether these observations are related to the elevated c-*myc* and c-*ras* expression in some cervical carcinomas (Riou et al. 1985).

RNA transcription and regulation

We have characterized the structures of HPV types 6 and 11 mRNAs by electron microscopy of RNA:DNA heteroduplexes (Fig. 1B) (Chow et al. 1987a) and those of HPV types 11, 16, and 18 by cDNA analysis (Nasseri et al. 1987; M.O. Rotenberg et al., in prep.; D. Palermo-Dilts et al., unpubl.). The critical polycistronic E2 mRNA on which this study is centered originates from a highly conserved TATA motif at the beginning of the early

region, the E6 promoter, which is characteristic of all HPVs trophic for the genital tract. It spans the E6 and E7 ORFs and, shortly into the E1 ORF, is spliced to a site just upstream from the E2 ORF and continues to the early polyadenylation site just downstream from the E5 ORF (Fig. 1B, e). Although encoded by the third major ORF in the spliced message, the E2 protein is synthesized in various mammalian cell lines (M.O. Rotenberg et al., in prep.). An E2-C mRNA (Fig. 1B, g) derived from a promoter in the middle of the E1 ORF has a short 5′ exon with the initiation codon spliced to a major exon coding for the carboxy-terminal half of the E2 ORF (E2-C). Similar mRNAs for HPV-16 and HPV-18 are predicted based on genomic DNA sequence homology and conservation of splice sites and coding capabilities. Multiple forms of E2 proteins have also been reported for bovine papillomavirus type-1 (Lambert et al. 1987; Hubbert et al. 1988). Both E2 and E2-C proteins regulate the enhancer and E6 promoter (Spalholz et al. 1985; Hirochika et al. 1987; Lambert et al. 1987; Phelps and Howley 1987; Chin et al. 1988 and in prep.). We have dissected the HPV-11 regulatory sequences in the URR and have shown that it consists of three components, a set of sequences responsive to E2 or E2-C proteins (E2-RS), a constitutive enhancer I (CEI), which has no cell-type specificity, and a constitutive enhancer II (CEII), which functions only in epithelial cells of human origin (Fig. 2) (Hirochika et al. 1987; Chin et al. 1988 and in prep.). We and other investigators (Androphy et al. 1987; Giri and Yaniv 1988; McBride et al. 1988; Moskaluk and Bastia 1988) have shown that the DNA-binding domains of E2 and E2-C proteins of human and animal papillomaviruses are in the common carboxy-terminal portions. Each recognizes the E2-RS with a sequence motif $ACCN_6GGT$, which occurs several times in the URR of all papillomavirus types, both human and animal, accounting for enhancer regulation by heterologous E2 proteins (Hirochika et al. 1987). In the mucosotrophic HPVs, two tandem copies (Fig. 2, nos. 3 and 4) of the E2-responsive sequence immediately precede the E6 promoter TATA motif, and a third copy (Fig. 2, no. 2) is further upstream near CEI. Purified HPV-11 E2 and E2-C proteins expressed in *Escherichia coli* can bind to each copy, and their DNAse I footprints extend beyond the core motif (Chin et al. 1988; Hirochika et al. 1988). When the tandem E2-responsive sequences (Fig. 2, nos. 3 and 4) are occupied in the absence of CEII, E2 protein acts as a transcriptional repressor rather than as an activator (M.T. Chin et al., in prep.), presumably because most of the TATA motif is occluded and unable to bind host transcription factor TFIID (Sawadogo and Roeder 1985). If the TATA motif is located further away from E2 responsive sequences, as it is in the human and animal papillomaviruses trophic for cutaneous skin or in recombinant plasmids containing the HPV URR linked to the SV40 promoter, the full-length E2 protein acts as a transcriptional *trans*-activator (Thierry and Yaniv 1987; Chin et al. 1988). The unique amino-terminal portion of each of the full-length E2 proteins has a highly conserved amphipathic, acidic α-helical domain analogous to those of many prokaryotic and eukaryotic transcription factors and is believed essential for protein-protein interactions in the formation of active transcription complexes. E2-C proteins invariably act as repressors, presumably by competing for E2-responsive sites without bringing in the activating domain (Cripe et al. 1987; Lambert et al. 1987; Chin et al. 1988).

The incorporation of tandem copies of synthetic CEII sequences 38-bp long into a URR deletion mutation lacking the native CEI and CEII partially abrogates E2 repression of the E6 promoter (M.T. Chin et al., in prep.). Similarly, multiple copies of a restriction fragment containing CEI also overcome the E2 repression and constitute a strong E2-independent enhancer in different cell types (Hirochika et al. 1988; M.T. Chin et al., in prep.). In cervical carcinoma cell lines, we have identified a ~44,000-dalton protein that binds to CEII.

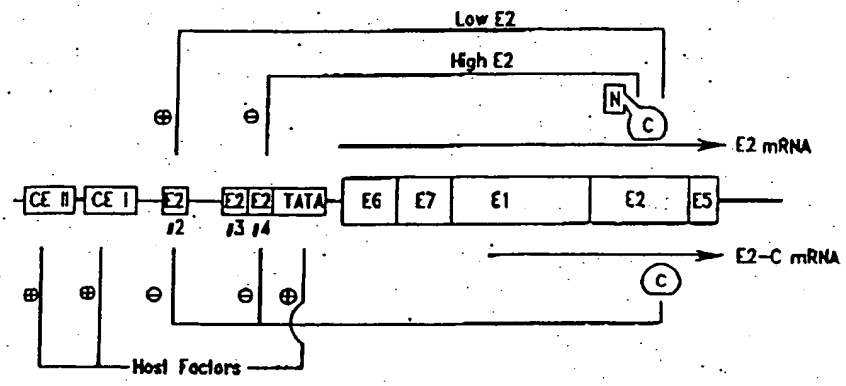

Figure 2 Regulation of early region transcription of HPVs trophic for the genital tract. Most of the features are known from studies of HPV-11 (Chow et al. 1987a; Hirochika et al. 1987, 1988; Chin et al. 1988 and in prep.). Cellular enhancer elements I (constitutive) and II (epithelial cell specific) vary among HPV types. HPV types 6, 11, 16, 18, 31, 33, and 7 all have tandem E2-responsive sequences (copies *3* and *4* for HPV-11, used as a model here) adjacent to the E6 promoter TATA motif and have another (copy *2*) near the cellular enhancer elements. The structures of the polycistronic E6-E7-E2-E5 and the E2-C-E5 mRNAs are shown. Both encode the carboxy-terminal DNA-binding domain of the E2 proteins, but only the full-length E2 transcript specifies the amino-terminal activation domain. The positive and negative effects of various host and viral transcriptional regulatory proteins are indicated by + and −, respectively, with the E2 protein being the key to the feedback circuit. The thin line in each mRNA represents an intron.

Although we have found that purified E2 and E2-C proteins expressed in *E. coli* have essentially equal affinities for the different copies of the E2-RS in the absence of other cellular factors, we speculate that E2 protein may preferentially bind to E2-RS 2 (Fig. 2) in association with these host factors, leading to the activation of the E6 promoter. Upon continued expression of the operon, we propose that E2 protein levels rise to a concentration sufficient to occupy E2-RS 3 and 4 (Fig. 2), blocking the TATA motif and down-regulating its own production to a low, steady state. In doing so, we believe that E6, E7, and possibly E5 transforming protein concentrations are also controlled. We also postulate that this feedback mainly occurs in the less differentiated basal and parabasal cells in which the CEI- and CEII-binding proteins might be limiting, thus preventing premature killing of the infected stem cells. This autoregulation is presumably overcome by increased concentration of these host factors in the differentiated keratinocytes, allowing the virus to proceed into productive infection. Such coordinate positive and negative feedback regulation is a hallmark of the bacteriophage λ repressor gene and of the *E. coli* arabinose operon, for instance, but this would appear to be one of the first candidates in a eukaryotic transcription unit. The balancing role played by E2-C repressor in this regulation is not yet clear. E2 proteins expressed in *E. coli* form dimers, including E2:E2-C heterodimers (McBride et al. 1988), potentially adding another level of fine tuning that can only be evaluated when regulation of the E2-C promoter is understood.

In situ hybridization

Exploration of the association between viral gene expression and cellular differentiation demands the ability to discern topographical differences of transcriptional activity in the context of tissue morphology. Such information is invariably lost by mass tissue analysis. The amount of tissue necessary for such biochemical analysis is relatively large and is further complicated by the number of viral types, viral genes, and host genes that need to be examined. Furthermore, pathological examination cannot be performed on the same tissue that is analyzed biochemically. In situ hybridization is the only technology that can address each of these problems. It permits the sensitive detection of specific mRNA and DNA sequences in recent and archival, formalin-fixed biopsies, while preserving the histology of the tissue and revealing the exact cellular and subcellular location of the sequences under study. Over the past 3 years, these methods have been increasingly utilized to address the molecular association of HPVs with neoplasia, as well as being recognized as one of the most powerful diagnostic and research tools available in anatomic pathology.

HPV cross-reactive and type-specific probes

We have described previously DNA:DNA heteroduplex mapping of genital HPVs and shown that the L1 ORFs are the most highly conserved regions, whereas the E5 region and the URR-E6-E7 segment are most unique to each type (Broker and Chow 1986; Chow et al. 1987b). On the basis of these data, subgenomic probes that can either serve as papillomavirus group cross-reactive probes or as type-specific probes were designed and confirmed by application to Southern transfer blots of DNA prototypes (Chow et al. 1987b; see also Manos et al., this volume). Single-stranded RNA probes generated from these clones were used to examine a wide variety of patient biopsies by in situ hybridization to conventional formalin-fixed, paraffin-embedded serial sections with a high sensitivity of detection. They generally reveal foci of infection by a single HPV type, but occasionally two types may be present, usually coinfecting the same clusters of cells, as though one of the viruses may provide a helper effect for the other.

An example of cross-reaction of RNA probes corresponding to the whole viral genomes of the closely related HPV-16 and HPV-31 (Lorincz et al. 1986; Chow et al. 1987b) is shown in Figure 3. Even at $T_m - 5°C$, HPV-31 whole genomic probes yield a weak positive signal with HPV-16 RNA present in a biopsy of a CIN I. When a subgenomic probe of HPV-16 from the relatively unique URR-E6-E7 region was used, a strong signal was maintained, whereas an HPV-31 E2-C-E5-L2-N type-specific probe did not cross-react. Similar type-specific RNA probes of HPV types 6, 11, 16, 18, and 31 (Chow et al. 1987b; M. Stoler et al., in prep.) have been used to identify and type papillomaviruses in a large number of lesions using in situ hybridization. This paper focuses on lesions containing HPV-16 and HPV-18.

HPV-16 mRNA expression revealed by in situ hybridization with exon-specific probes

There is only limited information on the precise structures of HPV-16 mRNAs, predominantly from S1 nuclease mapping of E6-E7 early region transcripts recovered from CaSki cells (Smotkin and Wettstein 1986), a cervical carcinoma cell line known to contain several hundred copies of full-length HPV-16 DNA integrated in tandem into several host chromosomes. Nonetheless, the similarity of genomic organization of all papillomaviruses and the conservation of splice donors, splice acceptors, and polyadenylation sites evident in the HPV-16 DNA sequence suggest that the mRNA structures are likely to be analogous to transcripts of HPV types 6 and 11 (Chow et al. 1987a). The exception is the presence of small introns in the E6 transcripts of HPV-16 and HPV-18 as well (Schneider-Gädicke and Schwarz 1986; Smotkin and Wettstein 1986). Using polymerase chain reactions on cDNAs prepared from RNA isolated from CaSki cells, we have identified a common splice donor (nt 226) and two alternative splice acceptors (nt 409 and nt 526) in HPV-16 transcripts. Both species lead to truncation of E6 protein by changing into reading phases closed shortly after the splice acceptor site (D. Palermo-Dilts et al., unpubl.).

We have cloned subgenomic regions of HPV-16 corresponding to the anticipated mRNA exons (Fig. 1A). These were placed in dual promoter vectors from which

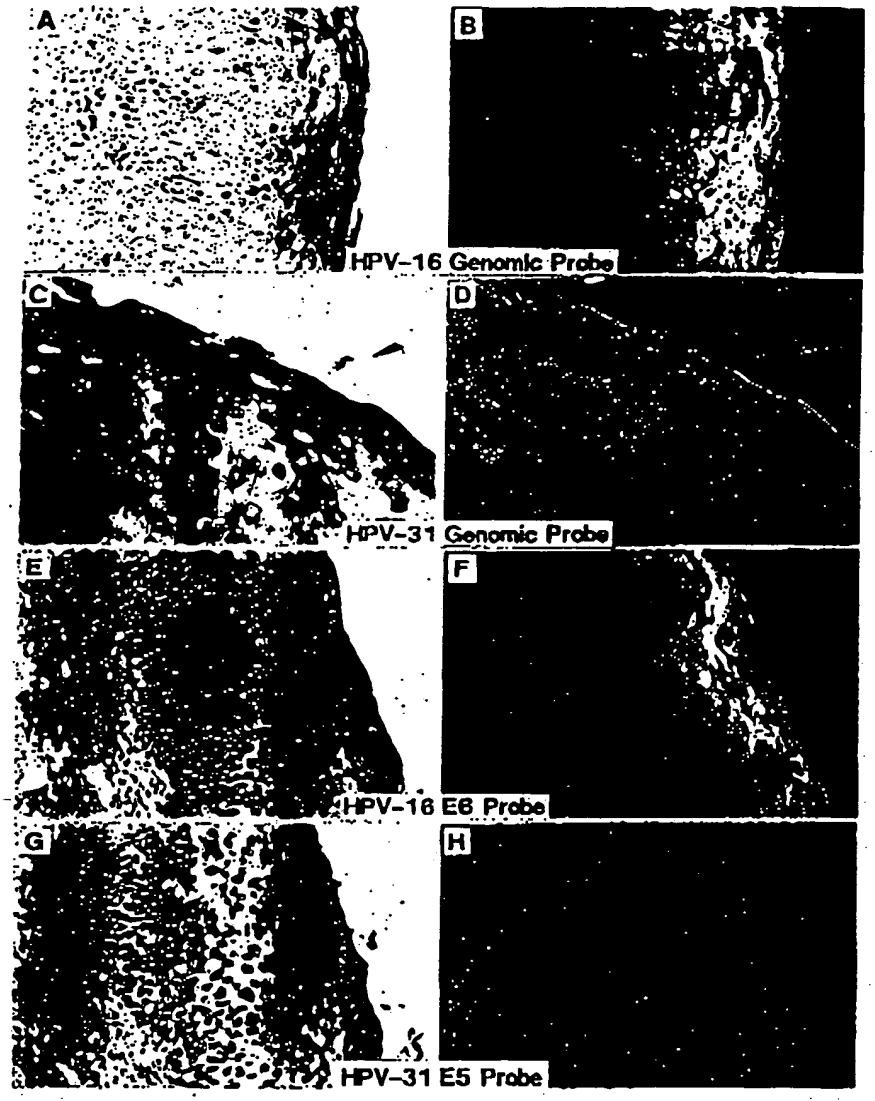

Figure 3 HPV-16 type-specific probes. ^{3}H-labeled antisense riboprobes complementary to viral RNA were generated from full-length genomic HPV-16 or HPV-31 DNA or from a subgenomic HPV-16 URR-E6-E7 (nt 7466–884) or subgenomic HPV-31 fragment spanning the region from E4/E2-C through the amino-terminal portion of the L2 ORF (nt 3362–4584). They were hybridized to adjacent sections of a biopsy diagnosed as CIN I. The sections exhibit HPV RNA signals when either whole genomic probe was employed. In contrast, only the HPV-16 RNA signals are evident when subgenomic, type-specific probes were applied.

either sense-strand or antisense-strand RNA probes can be generated by in vitro transcription. Tritium-labeled sense-strand RNA probes can be hybridized to denatured viral DNA in lesions without risk of confounding hybridization to viral mRNA of the same polarity. Conversely, ^{3}H-labeled antisense RNA will anneal with high efficiency and specificity to viral RNA in samples not subjected to prior denaturation of the DNA. Thus, the DNA and RNA distributions in specimens can be clearly distinguished.

Serial sections of biopsies of various cervical dysplasias and carcinomas were processed for in situ hybridization to probe for viral DNA or RNA, as described previously (Stoler and Broker 1986). Sections were first screened with HPV types 6, 11, 16, 18, and 31 type-specific probes. Hybridization was for 12–16 hours at $T_m - 25°C$; excess probe was eliminated by digestion with ribonuclease and high-stringency washing at $T_m - 5°C$. Sections were overlaid with liquid photographic emulsion and autoradiographed for 4 weeks. Following development, the slides were observed and photographed by dark-field illumination, which produces dramatic light scattering from ^{3}H-exposed silver grains and hence increases the sensitivity of detection. Signals are localized to the nucleic acid source: DNA and RNA precursors or processing by-products are in the nuclei, whereas mRNAs are predominantly in the cytoplasm.

Specimens containing HPV-16 that are described here include one case of koilocytotic atypia/CIN I, one case of CIN III, a set of three biopsies from a single

patient (representing squamous carcinoma in situ and endocervical adenocarcinoma in situ from a cone biopsy, as well as invasive adenosquamous carcinoma in the uterine corpus) and a second invasive squamous carcinoma. Together, these represent a nearly complete pathological spectrum. A set of serial sections of each specimen were challenged with mRNA exon-specific probes specific for E6-E7, E1, E2, E4(E2-C)-E5, L2 and L1 ORFs. The amounts of probes used were normalized according to size so that the signal intensities represent relative copy numbers of the target RNA species. Regions of each slide showing the same histological features were photographed and also semiquantitatively evaluated by photodensitometry of light scattering. One section of each specimen was hematoxylin plus eosin stained for histopathological evaluation. A subset of the autoradiographs from this study are presented in Figures 4, 5, and 6.

All early as well as late exons were abundant in the CIN I, with a distribution identical to that typically seen in HPV-6- or HPV-11-associated vulvar condylomas (M. Stoler et al., in prep.). E6-E7 and E4-E5 exon signals first appeared just above the basal cell layer of the epithelium (Fig. 4B,E). There was a dramatic increase in signal intensity in the more differentiated keratinocytes. This is the same transition point where viral DNA copy (replication) also becomes abundant (data not shown). One clear and perhaps significant difference between this HPV-16 CIN I and HPV-6 and HPV-11 vulvar condylomas is that the HPV-16 E4-E5 exon signal is only 2–3 times more abundant than the E6-E7 exon signal, whereas it is 10–20 times more abundant in HPV-6 and

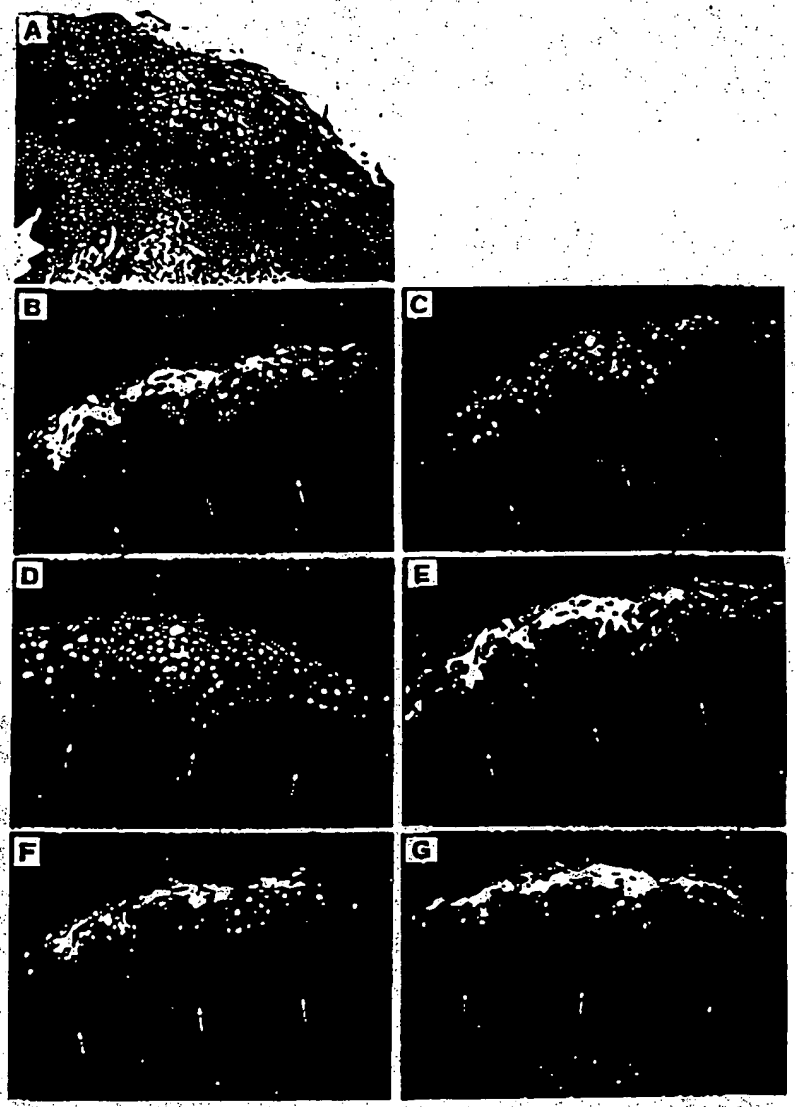

Figure 4 HPV-16 exon-specific probes of CEN I. (*A*) Histopathology (H & E stain), showing low-grade dysplasia and superficial koilocytes, visualized in bright-field. (*B–G*) Dark-field visualization of exposed silver grains in biopsies subjected to in situ hybridization with ^{3}H-labeled antisense RNA probes. Basement membranes are marked with arrows. (*B*) E6-E7; (*C*) E1; (*D*) E2 (note the nuclear localization of signals in *C* and *D*, presumably residual introns and primary transcripts); (*E*) E4-E5; (*F*) L2; and (*G*) L1.

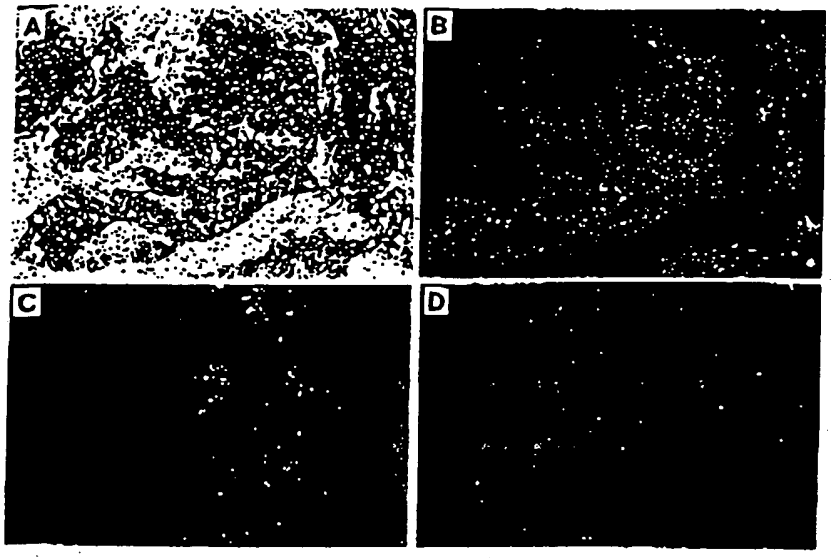

Figure 5 HPV-16 exon-specific probes of invasive adenosquamous carcinoma of the uterine corpus. (*A*) Histology; (*B*) E6-E7; (*C*) E4-E5; and (*D*) L1.

HPV-11 infections. This might suggest that the putative promoter immediately preceding the E1 ORF of HPV-16 is less active than that of HPV-6 and HPV-11. As with HPV-6 and HPV-11, the E1 and the E2 signals were almost exclusively nuclear (Fig. 4C,D). We attribute these to unprocessed primary transcripts and to residual intron material derived from the E6-E7-E4-E5 or E6-E7-E2-E5 mRNAs. Cytoplasmic signals from E1 or E2 were low or negligible. Cytoplasmic L2 and L1 ORF signals emerged only in the highly differentiated superficial strata (Fig. 4F,G), consistent with their synthesis as late messages encoding structural proteins. Occasional nuclear L2 and L1 signals in mid-epithelium may represent the 3′ run-on of early transcripts processed at the early polyadenylation site or perhaps are precursors to late messages. The tissue, though typically hyperproliferative, showed full differentiation into stratified epithelium as well as koilocytotic atypia indicative of a HPV cytopathic effect.

The higher grades of lesions exhibited dramatically less viral transcription and also were much more restricted in cellular differentiation. Nonetheless, it is important to recognize that the amounts of viral RNA for the degree of differentiation were of similar or higher abundance compared with RNA concentrations in the basal and parabasal cells of equivalent differentiation in the CIN I. These observations are critical to the proposed mechanisms of HPV carcinogenesis to be de-

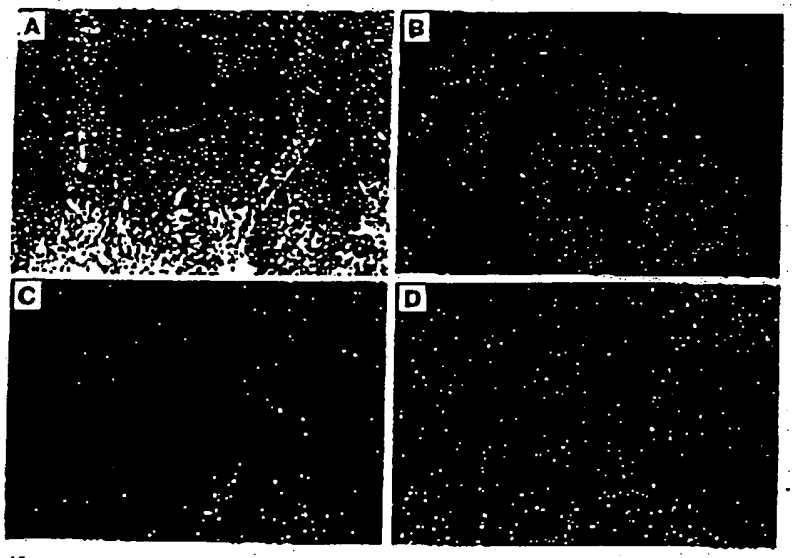

Figure 6 HPV-16 exon-specific probes of invasive squamous carcinoma of the cervix. (*A*) Histology; (*B*) E6-E7; (*C*) E4-E5; and (*D*) L1.

scribed. As might be anticipated, based on a requirement for terminal differentiation, late RNA (L2 and L1) was generally not made in any of the higher-grade lesions. The CIN III exhibited modest amounts of E6-E7 and E4-E5 exons in the cytoplasm and proportionally lower E1 and E2 signals in the nuclei (data not shown). L2 and L1 exons were absent. Similarly, the histologically distinct squamous carcinoma in situ, adenocarcinoma in situ, and invasive adenosquamous carcinoma from a single patient all had the same exon transcription patterns, suggestive of a clonal origin for these tumor types. Within this set of specimens and others we have investigated, E6-E7 signals were clearly more abundant than E4-E5 signals (Fig. 5). One interpretation is that some of the viral DNAs are integrated near the E1/E2 junction, dissociating the E2-E4-E5 region from their promoters, whereas others remain episomal. As described above, we believe that the full-length E2 protein in the less-differentiated cells acts both as an activator and as a repressor for the E6 promoter. Upon deletion or disruption of the E2 and E2-C genes, the E6 promoter would be derepressed, which could account for the higher relative E6-E7 transcription compared with E4-E5 transcription. Consistent with this hypothesis was our finding of a purely integrated pattern of gene expression that exhibits substantial levels of E6-E7-E1 signals but no E2-E4-E5, L2, or L1 signals (Fig. 6 and data not shown) in a second case of invasive cancer.

We have noticed in several similar cases that the L1 exon is anomalously expressed, despite the absence of E4-E5 and L2 signals. We infer that the L1 RNA (Fig. 1B, i) did not arise from its usual promoter, but rather from an upstream host gene promoter, and is part of a transcript that either runs into the integrated HPV DNA or is spliced to the L1 acceptor site. Termination would be expected at the L1 polyadenylation signal. This suggestive evidence for a host-L1 fusion transcript could also provide an explanation for the successful processing of the E6-E7 messages initiated from integrated viral genomes. They, like most eukaryotic messages, probably require splicing for transport to the cytoplasm and polyadenylation for stability. We speculate that the presumptive, interrupted host cell gene downstream from the integrated viral genome provides the 3′ exon(s) and the polyadenylation signal allowing the completion of a E6-E7-host fusion transcript. In brief, it seems as though the HPV DNA integration event leading to carcinogenic progression may require the chance insertion into a class of genes that is expressed in epithelial cells. This proposal is being tested experimentally.

HPV-18-associated lesions have also been examined with analogous HPV-18 exon-specific probes (Fig. 7).

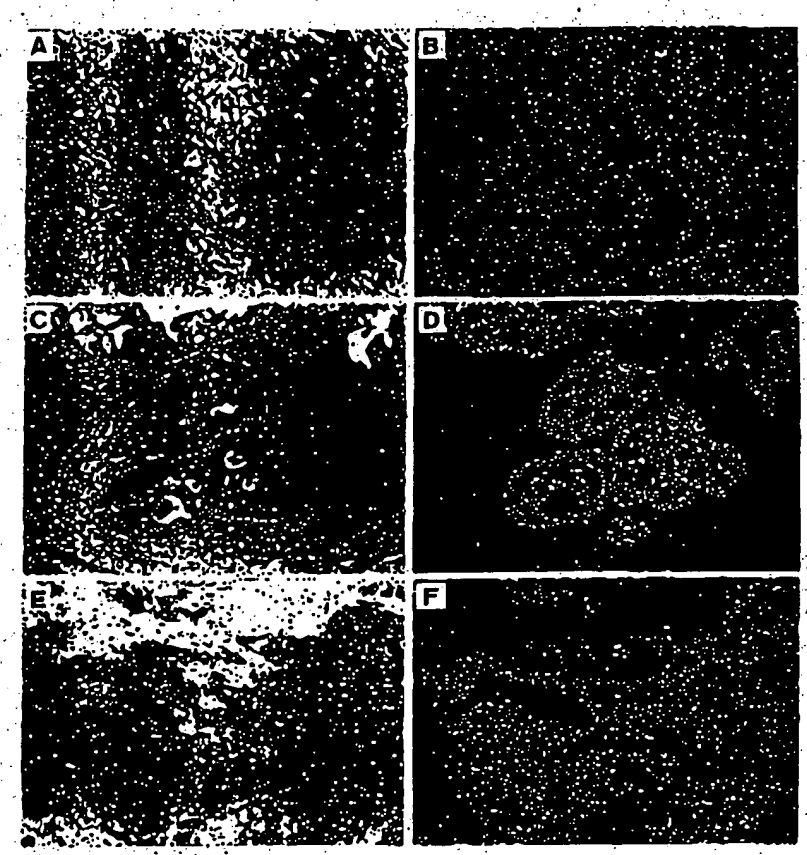

Figure 7 HPV-18-associated carcinomas. Bright-field and dark-field pairs are shown. Following identification with type-specific probes, whole genomic antisense probes were used for greater sensitivity. In each tumor, the total RNA signals are located in invading epithelial cells surrounded by negative stromal fibroblasts. (*A,B*) Squamous carcinoma of the exocervix; (*C,D*) adenocarcinoma of the endocervix; (*E,F*) small-cell neuroendocrine carcinoma of the endocervix.

One case of an HPV-18 CIN I expressed all early and late mRNAs (C. Rhodes et al., unpubl.). HPV-18 has previously been found predominantly in adenocarcinomas and adenosquamous carcinomas (Tase et al. 1988; Wilczynski et al. 1988). We have recently discovered that small cell neuroendocrine carcinomas are highly correlated with the presence and expression of HPV-18 genes (M. Stoler, unpubl.). All carcinomas exhibited a transcription pattern indicative of integration (C. Rhodes et al., unpubl.). The absence of cases with full early region transcription (particularly the E2-E4-E5 region) analogous to some of the HPV-16 CIN III or carcinomas, points to a high probability of rapid HPV-18 DNA integration and loss of the E2-E4-E5 genes. This is entirely consistent with the more aggressive nature of HPV-18 carcinomas relative to those containing HPV-16 DNA (Kurman et al. 1988).

Summary model of HPV carcinogenesis

On the basis of the described transcriptional and regulatory data from experimentation in vitro and in vivo, we propose the following molecular mechanism for HPV carcinogenesis. One of the key elements to the model is that the E2 transcription regulatory protein is translated from a polycistronic transcript that is derived from the E6 promoter and that also encodes several transformation proteins from the E6 and E7 ORFs. Low levels of E2 protein, in association with host transcriptional factors, can up-regulate transcription from the E6 promoter. We hypothesize that the expression of such host factors depends on the state of cellular differentiation. In the mucosotrophic papillomaviruses, a tandem pair of E2 and E2-C protein-binding sites are located adjacent to the E6 promoter TATA motif, which is partially occluded by binding of either E2 protein. Such interference eventually overcomes autostimulation and down-regulates the production of the polycistronic message, providing feedback control to E2 and the viral transforming proteins. This balance leads to a low level of viral maintenance in relatively undifferentiated keratinocytes. As the epithelium differentiates, increases (or other changes) in host regulatory proteins, for instance in factors like CEI- and CEII-binding proteins that can overcome E2 repression, may relieve the negative regulation and lead to high levels of early transcription and to viral DNA replication. Increased gene dosage results in higher viral gene expression and the chance for breakthrough to late gene expression and virion production. That cellular transformation does not occur at this stage can be attributed to the fact that these differentiated cells have already lost the ability to divide. In some rare events, the steady maintenance state in the infected stem cells is disrupted. Mutations in the HPV URR or stimulation by other viruses could up-regulate viral early gene expression. Alternatively, mutations in viral genes required for autonomous replication or mutagenic events resulting in viral and host DNA breakage may lead to integration. When integration interrupts the expression of the E2 proteins, the viral transformation proteins would be derepressed, provided the integration happened to occur into active chromatin in a genetic region that could provide downstream RNA splice and polyadenylation sites. In such cases, the dividing basal and parabasal cells would be subject to unusual and excessive amounts of these transforming proteins. Excessive E7 protein stoichiometrically could sequester the RB gene product (Dyson et al., this volume) and perhaps other anti-oncoproteins and release the cells from controlled growth, initiating carcinogensis. Clearly, there are numerous untested aspects of this proposal, but a tantalizing profile of many of the components has been provided through efforts in a number of laboratories studying RNA transcription and processing, enhancer-promoter regulation, protein structure and function, and gene expression in relation to tissue pathogenesis. Without doubt, there are other critical host cell-virus interactions essential for initiation and maintenance of the transformed state and for invasion and metastasis, which will also be the subject of future observation and experimentation.

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Melvyn Greaves
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Review

Molecular events in uterine cervical cancer

Shirley A Southern, C Simon Herrington

Objective: To review the literature regarding the molecular events which occur in the development of uterine cervical cancer, with particular reference to human papillomavirus (HPV) infection.
Methodology: Bibliographic searches of Medline and the ISI citation databases using appropriate keywords, including the following: papillomavirus, cervix, pathology, cyclin, chromosome, heterozygosity, telomerase, smoking, hormones, HLA, immune response, HIV, HSV, EBV.
Conclusions: It has become clear that most cervical neoplasia, whether intraepithelial or invasive, is attributable in part to HPV infection. However, HPV infection alone is not sufficient, and, in a small proportion of cases, may not be necessary for malignant transformation. There is increasing evidence that HPV gene products interfere with cell cycle control leading to secondary accumulation of small and large scale genetic abnormalities. This may explain the association of viral persistence with lesion progression but, in many patients, secondary factors, such as smoking and immune response, are clearly important. However, the mechanisms involved in the interaction between HPV and host factors are poorly understood.
(*Sex Transm Inf* 1998;74:101–109)

Keywords: cervix; carcinoma; papillomavirus; aetiology; molecular biology

Introduction

Human papillomaviruses (HPV) have been identified as the major aetiological factor in cervical carcinogenesis.[1] Epidemiological evidence indicates that the majority of cervical neoplasia is attributable to HPV infection but, although certain HPV genes are capable of immortalisation and can cooperate in the process of transformation, not all non-invasive lesions progress to the full malignant phenotype indicating that other cofactors are required.[2] This review considers the molecular biology of cervical neoplasia, particularly that of the HPVs, the interaction of HPVs with epithelial cells, and the influence of possible associated cofactors. The combination of such factors leads to a consortium of molecular events involved in the evolution of intraepithelial and invasive disease (see fig 1).

Human papillomaviruses and cervical neoplasia

VIRAL STRUCTURE

Papillomaviruses are small DNA viruses approximately 55 nm in diameter. Mature viral particles have an icosahedral outer capsid coat composed of two structural proteins. One of these (the L1 protein) comprises 80% of the total viral protein and has a relative molecular mass (M_r) of 53 000–59 000. The other (the L2 protein) is a minor component, and has a M_r of 70 000. Contained within the capsid is the viral genome which is a double stranded circular DNA approximately 7.9 kilobases (kb) in length.[1] The molecular organisation is similar for all 78 different types of HPV which have so far been isolated. Each genome can be divided into early (E) and late (L) regions, containing seven early and two late open reading frames (ORFs), and a non-coding region, referred to as the upstream regulatory region (URR). Expression of the early genes occurs at the onset of infection, and the products of these genes mediate specific functions controlling viral replication and, in the case of the oncogenic viruses, cellular transformation. The E1 gene is involved in viral replication and genome maintenance.[4] The E2 gene is a transcriptional regulator[5] and is also involved in viral DNA replication.[4] The E4 gene encodes several proteins which disrupt the cytoplasmic keratin network.[6] This produces the classic cytoplasmic halo effect known as koilocytosis. The E5 gene may play a role in cellular transformation by its interactions with cell membrane growth factor receptors.[7] The E6 and E7 genes, which lie immediately downstream of the URR, encode the major transforming proteins which are capable, under appropriate conditions, of inducing cell proliferation, immortalisation, and transformation.[1] Finally, the L1 and L2 ORFs encode for the viral protein coat and are activated towards the final stages of the viral cycle, and hence within superficial, terminally differentiated cells.[8] The L1 gene is frequently used for HPV typing[9 10] and encodes the common papillomavirus antigen which is targeted by antibodies used in the immunohistochemical detection of productive HPV infection.

CLASSIFICATION OF HPVS

HPVs were originally classified according to their degree of solution phase homology.[11] With the advent of widespread polymerase chain reaction (PCR) amplification and sequencing, classification is now usually based on partial sequencing of 399 bp in the E6 region and/or 291 bp in the L1 region of the virus.[12] A new HPV type is assigned when there is less than 90% homology with other previously typed HPVs, a subtype being 90–98% homologous and a variant >98%.[12] A number of phylogenetic trees have been generated using this

Department of Pathology, University of Liverpool, Royal Liverpool University Hospital, Daulby Street, Liverpool L69 3GA
S A Southern
C S Herrington

Correspondence to:
Dr C S Herrington.

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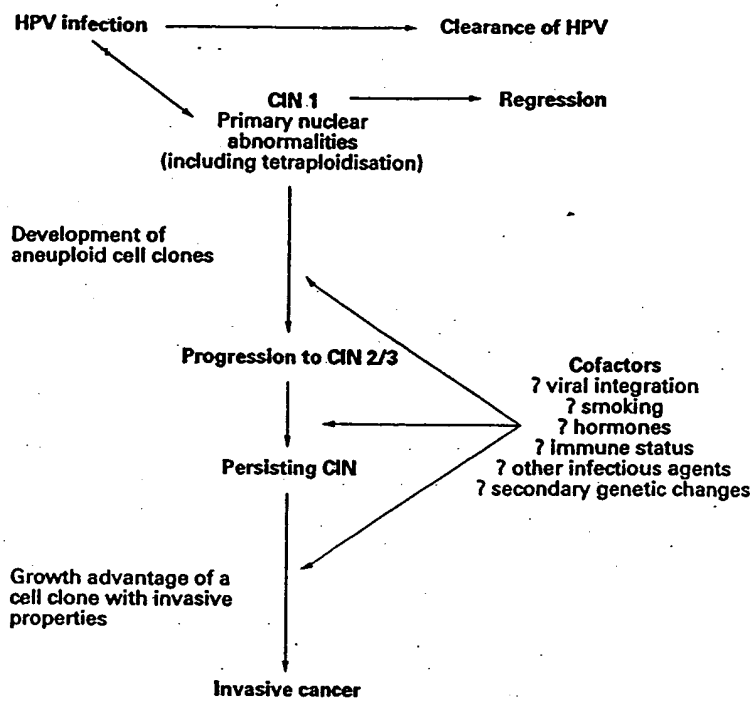

Figure 1 Schematic overview of the human papillomavirus (HPV) associated molecular events involved in cervical carcinogenesis (CIN = cervical intraepithelial neoplasia).

system but a more recent classification of HPVs based on clinicopathological associations as well as genetic structure gives a better view of both functional and genetic properties.[13] This system provides a more robust basis for the investigation of clinical associations and biological properties of these viruses. For example, recent studies have demonstrated not only that HPV 16 variants are widely prevalent in cervical cancers[14] but also that such variants differ in their ability to interact with host cell mechanisms.[15]

CLINICAL ASSOCIATIONS OF HPV GENOTYPES

Molecular cloning of viral nucleic acids, amplification by PCR and sequencing have demonstrated over 78 different HPV genotypes, at least 30 of which have been detected in the genital tract.[13 16] These HPVs have been divided into low, intermediate, and high risk types according to their segregation with the different grades of intraepithelial and invasive disease. Low risk HPV types (for example, HPV 6, 11, 40, 42, 43, 44) are usually associated with benign exophytic genital warts. HPV 6 and 11 are present in over 90% of condylomas, with about two thirds of these containing HPV 6 and one third HPV 11. They are also associated with low grade squamous intraepithelial lesions (SILs) (wart virus change and CIN 1) but are only rarely found in high grade SILs (CIN 2 and 3) and invasive carcinomas. By contrast, intermediate (particularly HPV 31, 33, 39, 52, and 58) and high risk (HPV 16, 18, 45, and 56) HPV types are associated with "flat" condylomas, all grades of SIL and invasive carcinoma.[17 18] The concept of high, intermediate, and low risk HPV is supported by many epidemiological case controlled studies which have collectively shown a consistent association between high and intermediate risk HPV and high grade cervical disease. In a worldwide study of more than 1000 specimens from patients with invasive cervical cancer HPV DNA was identified in 93% of tumours. HPV 16 was present in 50% of the specimens, HPV 18 in 14%, HPV 45 in 8%, and HPV 31 in 5%. In squamous cell tumours HPV 16 predominated (51%) but HPV 18 was the commonest type found in adenocarcinomas (56%) and adenosquamous tumours (39%).[19] Another study involving cervical specimens from 2627 women showed HPV present in 79.3% of women with definite cervical neoplasia (627 of 791) and in 23.7% of patients with borderline atypia. Low risk HPV (HPV 6, 11, 42, 43, and 44) was present in 20.2% (76 of 377) of low grade lesions but absent in all cancers. Intermediate risk HPV (HPV 31, 33, 35, 51, 52, and 58) was detected in 23.8% (62 of 261) of high grade lesions but in only 10.5% (16 of 153) cancers. HPV 16 was associated with 47.1% of both high grade SILs (123 of 261) and cancers (72 of 153) and HPV 18, 45, and 56 were found in 26.8% (41 of 153) of invasive carcinomas but only 6.5% (17 of 261) of high grade SIL.[17]

The risk of progression from low grade to high grade SIL is greater in patients with persistent HPV infection,[20 21] and in those with high viral load[20] suggesting that prolonged exposure to viral effects is important in the pathogenesis of cervical disease. Whether this is related to viral factors, such as sequence variation, or is a reflection of host factors, such as immune status, remains to be determined.

Recently, a PCR based method for determining clonality has shown a strong correlation between HPV type and clonal status. Morphologically low grade SILs could be classed into two biologically distinct lesions. That associated with HPV types 16, 18, 31, 33, 35, 39, 45, 56, 58, or 65 was monoclonal indicating clonal expansion of infected keratinocytes, while that associated with other HPV types was polyclonal and probably represents non-neoplastic virally induced proliferation.[22] Whether the distinction between monoclonal and polyclonal HPV infection gives information additional to morphology and HPV type remains to be determined.

PATHOLOGY OF HPV INFECTION

HPVs are epitheliotropic by nature, infecting the cervical squamous epithelium possibly through small abrasions in the tissue. The ensuing virus life cycle is then closely linked to keratinocyte differentiation. In the proliferating basal epithelial cells, thought to be the site of initial infection, the viral genome is maintained as a low copy episome.[23] As the keratinocyte undergoes progressive differentiation, viral genome amplification and gene expression increase until late "L" gene expression and virion production occur in the terminally differentiated superficial cells. This form of infection leads to koilocytosis, nuclear enlargement, dyskeratosis, multinucleation, and in some cases low grade SIL. Such lesions may regress, persist, or progress.

A second form of infection (non-permissive transformable infection) occurs when viral

without a history of genital herpes, should be strongly advised not to have sex at the time of

replication and vegetative viral production does not occur and may be found in both squamous and glandular tissue; infection of reserve cells which are committed to glandular differentiation and which do not allow permissive infection results in either aborted or non-permissive transformable infection. Viral DNA persists as either an extrachromosomal element or by integration into the host DNA as a single copy or multiple head to tail tandemly repeated copies at many chromosomal fragile sites.[24] The site of integration into the host genome does not appear to be consistent, although late replicating regions are targeted,[25] suggesting that structural and functional factors may be important. The viral breakpoint is more consistent as integration often causes disruption of the viral E2 gene in a manner that results in loss of function.[26] Moreover, disruption of either the E1 or E2 gene can lead to enhanced immortalisation capacity.[27] Viral integration also precludes late gene expression, even if the late genes are retained in the integrated viral genomes.[28] The mechanisms of viral integration are not well understood, although it has been demonstrated that expression of E6 or E7 of HPV 16 increases integration of foreign DNA compared with HPV 6 or 11[29]: this is consistent with the observation that viral integration is rare in lesions infected with low risk HPVs.

Viral integration with associated disruption of the E2 gene and consequent removal of transcriptional repression is one mechanism of upregulation of E6/E7 expression. However, in one study, integration of HPV 16 was associated with increased stability of E6/E7 mRNA suggesting that alternative pathways may occur.[30] Moreover, episomal viral DNA is frequently present and amplified in cervical carcinomas,[31 32] and this amplification is dependent on retention of intact E1 and E2 genes in these lesions.[31] It has therefore been suggested that HPV amplification may provide an alternative mechanism for the upregulation of early gene expression in some tumours. These effects are clearly important in the process of squamous cell transformation as E6 and E7 expression is required for maintenance of the transformed phenotype.[33 34]

Several cellular transcriptional control factors are involved in the control of HPV early gene expression but the mechanisms involved are not well understood. It has been demonstrated that E6/E7 expression is dependent on expression of AP1, a cellular transcriptional control factor.[35] Interestingly, upregulation of the expression of c-jun (a component of AP-1) has been demonstrated in association with low risk HPV infection of condylomas so this pathway may be common to both high and low risk HPV types.[36]

EXPERIMENTAL HPV INDUCED TRANSFORMATION

HPV DNA, particularly the E6 and E7 genes, can immortalise primary cervical cells and human foreskin keratinocytes in culture[37 38] but transformation of normal cells generally requires cooperation between HPV and other oncogenic sequences, such as EJ-*ras*,[39] in keeping with other factors being involved in this process in vivo. Transfection of normal keratinocytes with HPV sequences can prevent cellular differentiation resulting in induction of morphological changes similar to CIN in raft culture.[40 41] Culture of naturally occurring lesions in a similar system leads to the production of morphological changes in vitro similar to those present in vivo.[42]

Fusion of HPV 18 containing HeLa cells with normal fibroblasts results in the repression of the malignant HeLa cell phenotype.[43] This system led to the localisation of a putative normal cellular factor, localised to chromosome 11, involved in the suppression of HPV E6/E7 expression. This was termed cellular interfering factor[44] but, to date, it has not been precisely identified. There is, however, evidence that a tumour suppressor gene important in cervical neoplasia may be located on chromosome 11 (see below).

Another approach to the study of HPV early genes involves the production of mice transgenic for the E6/E7 genes of high risk HPVs, particularly HPV 16. By introducing these genes under the control of the keratin 14 promoter, expression can be targeted to squamous epithelium and the effects of such expression analysed in vivo.[45 46] These mice develop hyperplastic and dysplastic squamous lesions but only progress to invasive malignancy when back crossed with certain strains, indicating that genetic background is important in the determination of susceptibility to invasive disease. This is in keeping with the effect of HLA genotype seen in human disease (see below).

HPV AND CELL CYCLE CONTROL

Recently, it has become increasingly clear that HPVs, like many other DNA viruses, achieve their replication by interference with normal cell cycle control mechanisms. Given that malignant transformation is also intimately related to these processes, it is likely that the oncogenic potential of papillomaviruses lies in their ability to alter cell cycle checkpoints, thereby leading to accumulation and transmission of genetic abnormalities. The initial observation that the E6 and E7 oncoproteins bind to the p53 and pRB proteins respectively, both of which are involved in the regulation of growth control, demonstrated that the HPV genes possessed functions analogous to those already known for SV40 and adenovirus.[47-49] Moreover, the E6 proteins of high risk HPVs, particularly HPV 16 and 18, bound more effectively to, and led to degradation of, the p53 protein via a ubiquitin mediated pathway,[50] indicating a functional difference between high and low risk HPVs. Introduction of mutations into HPV 11 and 16 E6 proteins by exchanging the p53 binding domains altered the ability of these proteins to bind to and degrade p53[51] and confirmed these differences. In addition, the E6 proteins of naturally occurring variants of HPV 16 differ in their abilities to bind to and degrade p53 protein,[15] indicating that these differences are determined by relatively small sequence variations.

p53 appears to protect the physical integrity of the genome by regulating the G1 cell cycle "checkpoint" preventing entry into S phase of cells containing DNA strand break damage, thus allowing DNA repair or apoptosis and avoiding replication of a damaged template.[52] DNA damage causes an increase in wild type p53 which activates expression of the WAF 1 gene whose protein product, p21 WAF, binds and inhibits cyclin/cyclin dependent kinase (CDK) activity, in particular CDK 2.[53] The inhibition of CDK 2 activity alters the phosphorylation state of the retinoblastoma gene product (pRB). Under normal cyclin/CDK control the RB protein is in the underphosphorylated form in G1 phase and becomes highly phosphorylated through the S and G2 phases. The underphosphorylated pRB readily complexes with the transcription factor E2F, but the phosphorylated form does not, allowing free E2F to activate transcription of a range of genes whose products are essential for cell cycle progression.[54] Therefore, temporary inhibition of the CDK 2 indirectly by p53 conserves the underphosphorylated pRB/E2F complex halting transcriptional activation and allowing DNA repair before entering the S phase. Cells expressing HPV 16/18 E6 and E7 lack this G1 cell cycle checkpoint. The resulting failure of the normal control system may ultimately contribute to the accumulation of genetic alterations required for tumour development and/or progression and lead to DNA instability which may facilitate integration of viral DNA into host chromosomes and further malignant progression.

Much of the control of the cell cycle is mediated by the cyclin proteins which regulate the activity of CDKs. Cyclin control immediately before and during S phase is mediated by cyclins E and A and the overexpression of these cyclins in association with HPV infection suggests that the virus can "take over" certain host cell cycle control mechanisms to facilitate viral, rather than cellular, DNA replication.[55] The cell is then held in a prolonged replication phase until degradation of the increased cyclin is completed. Cyclin D is responsible for cells passing through G1 phase and complexes and inactivates Rb protein in a similar fashion to HPV E7. Intuitively, therefore, E7 could circumvent cellular requirements for cyclin D expression: this appears to be the case in cervical neoplasia.[56]

All of the cell cycle events are intimately related to viral early gene expression and upregulation of E6 and E7, by whatever mechanism, is likely to lead to such cells having a growth advantage over their neighbours. All HPV infected cells, regardless of HPV type, undergo proliferation whether uncontrolled or self limited. However, HPV 16/18 infected cells lack G1 checkpoints[57] favouring viral integration and inducing genetic instability. This mechanism remains intact with HPV 6/11 in which the virus remains in the episomal form, in part due to the differences in interaction between p53 and the E6 proteins of HPV 16/18 and HPV 6/11.[51] The inability to integrate and an intact G1 checkpoint, with overexpression of cyclin/CDKs, could account for HPV 6/11 infected lesions proliferating while remaining benign.

In addition to affecting early cell cycle control, HPV 16/18 infection is associated with the presence of abnormal mitotic figures suggesting disruption of mitotic events.[58] Before M phase, cyclin B/p34 cdc 2 complex together forming mitosis promoting factor (MPF).[59] In the normal cell cycle, p34 cdc 2 (CDK 1) is at a constant level throughout, its activity being controlled by the altering levels of the complexing cyclin B and by phosphorylation. Dephosphorylation of MPF causes an increase in associated histone 1 kinase activity and initiation of mitosis.[60] Cyclin A also complexes with p34 cdc 2 and is required for the onset of mitosis.[61] Exit from mitosis is attributed to cyclin B degradation.[62] Increased expression and altered activity of these proteins by HPV[60] could contribute to mitotic defects and chromosomal aberrations. Indeed, overexpression of the HPV 16 E6 and E7 genes in the presence of mitotic spindle inhibitors has been shown to induce genome wide DNA rereplication without intervening mitosis.[63] Similarly, overexpression of the E2 gene alone leads to S phase arrest and reduplication of keratinocyte DNA content.[64]

Thus, HPV genes are capable of interfering with both G1/S and G2/M cell cycle checkpoints. Abrogation of the former may lead to accumulation of small scale genetic abnormalities while induction of abnormalities of mitotic control is more likely to lead to more gross genetic changes involving whole chromosomes (chromosomal instability). The differences between HPV types may well be reflected in their differing abilities to block these checkpoints in vivo.

Although the binding of high risk HPV E7 proteins to pRb may be involved in immortalisation and transformation owing to disturbances in cell cycle control, HPV 16 E7 has been shown recently to be capable of binding directly to members of the AP1 family of transcription factors.[65] As (i) the AP1 transcription factors play an important regulatory role in the differentiation of keratinocytes and (ii) the HPV life cycle is closely tied to cell differentiation, it was hypothesised that binding of Jun proteins by E7 may result in inhibition of the cell differentiation required for efficient virus replication. The E7 protein may therefore play an additional role in transformation which is independent of its ability to bind to pRb.

Cofactors in the evolution of cervical neoplasia

The epidemiological evidence that many HPV infections regress, and that progression of intraepithelial lesions is associated with viral persistence, taken in conjunction with the in vitro data that HPV genes are capable of immortalisation of normal cells, but not their transformation (see above), indicate that secondary changes are important in HPV associated cervical carcinogenesis. These changes may occur as a direct consequence of HPV infection or indirectly through the action of

cofactors, either innate or acquired. This is consistent with current multistage models of carcinogenesis, in which several genetic events are required to effect transformation.

GENETIC CHANGES

Genetic damage with consequent loss of tumour suppressor genes, or activation of cellular oncogenes, can lead to cellular immortalisation and transformation. Such genetic changes may occur as a result of cell cycle checkpoint abnormalities induced by HPV gene expression, or may be related to environmental factors such as the formation of smoking related DNA adducts. However, loss or mutation of conventional oncogenes and tumour suppressor genes is uncommon in cervical neoplasia.[2 66]

Conventional cytogenetic studies have demonstrated non-random chromosome abnormalities involving chromosomes 1, 3, 5, 11, and 17 in cervical carcinoma[67] but relatively few studies have examined the correlation between HPV and chromosome abnormalities in cervical cells and tissues. In a cell culture model involving transfection of high risk HPV DNA, immortalisation was associated with clonal allele loss on chromosomes 3p, 11q, 18q, and 10p as assessed by microsatellite loss of heterozygosity (LOH).[68] Similarly, comparative genomic hybridisation demonstrated abnormalities of chromosomes 3, 4, 10, and 11 in keratinocytes stably transfected with HPV 16.[69] Numerical abnormalities of chromosomes 11, 17, and X were identified in 88% of invasive squamous carcinomas of the cervix by interphase cytogenetics but there was no relation of these abnormalities to the type or morphological distribution of the HPV sequences present.[70]

Analysis of LOH in naturally occurring tumours has demonstrated changes in almost all chromosome arms,[71] but the most frequent abnormalities are present in similar chromosomes to those involved in the in vitro studies.[72–80] Several studies have shown LOH on the short arm of chromosome 3 in cervical carcinoma, particularly in the 3p13–21.1, 3p21, and 3p21–22 regions.[72–75] Using comparative genomic hybridisation, gain of chromosome 3q was identified in severe dysplasia/CIS and was overrepresented in 90% of carcinomas, suggesting that gain of this region was important in progression from non-invasive lesions to invasive carcinoma.[81] In other studies, frequent LOH has been identified on chromosome 17p,[73 76 77] chromosome 11q,[78 79] chromosome 4,[80] and chromosomes 6p and 18q.[75] Microsatellite instability, which is frequently associated with some tumour types—for example, colorectal carcinoma, does not appear to be common in cervical carcinoma, being identified in only 5.6% of tumours in one study.[82]

There is increasing evidence that cellular immortalisation requires restoration of chromosome telomere length by activation of telomerase.[83] Recently, it has been demonstrated that expression of the E6 protein of HPV 16 can induce telomerase activity but that this induction is not sufficient for keratinocyte immortalisation.[84] The observation that clonal allele loss on chromosomes 3p, 10p, 11q, and 18q was accompanied by telomerase activation in keratinocytes immortalised by HPV transfection[68] is in keeping with these findings. It is therefore not surprising that telomerase activity was identified in all of 10 cervical carcinomas reported in a recent study.[85]

Although there is an emerging pattern of genetic changes in neoplastic cervical lesions, little is know of the mechanisms of induction of these abnormalities, or of the identity of the genes involved. The relation between productive viral infection, viral gene expression, viral integration, keratinocyte differentiation, and DNA abnormalities is also poorly understood.

SMOKING

Epidemiological studies have shown an important correlation between cigarette smoking and the development of cervical cancer. A twofold increased risk of CIN and invasive disease has been demonstrated among smokers[86]; passive smoking has also been implicated[87] and cessation of cigarette smoking is associated with a reduction in the size of CIN lesions by approximately 20%.[88]

The actual mechanism by which smoking leads to an increased risk of cervical cancer is not fully understood but Langerhans' cell number is reduced in the cervical epithelium of smokers,[89 90] suggesting that abnormalities of local immune surveillance may be important. Alternatively, the constituents of smoke and their derivatives may interact directly with HPV. Nicotine and cotinine are frequently found at high levels in cervical mucus[91] and can induce proliferation of HPV transformed cervical cells.[92] Polycyclic aromatic hydrocarbons such as benz[a]pyrene present in cigarette smoke have been shown to inhibit cell proliferation in both "normal" and HPV 16 immortalised cervical cell lines, but with inhibition occurring at a 20-fold lower concentration in the normal cells.[93] Moreover, as these compounds can form adduction products with DNA, they are potentially genotoxic. This effect may be important in vivo as DNA adduction products were found to be present in normal cervical epithelium at a higher level in smokers than in non-smokers.[94]

OTHER INFECTIOUS AGENTS

It has been suggested that more than one infectious agent may act in a synergistic manner in the development of cervical cancer but there is little evidence for the interaction of HPV and infectious agents other than herpes simplex virus (HSV)[95] and HIV. Experimental evidence has also shown that cells transformed by HPV 16/18 are capable of inducing tumours in mice only after transfection with HSV 2.[96] Moreover, women testing seropositive for HSV 2 alone had a risk ratio of 1.2 for cervical cancer, but those with HPV 16/18 in addition had a risk ratio of 8.8; in those with HPV 16/18 alone the risk ratio was 4.3.[97]

In HIV positive patients who are not immunocompetent there is an increased incidence of

HPV infection with associated epithelial cell abnormalities.[98] In addition, the HIV *tat* 1 protein is capable of transactivating HPV 16 transcription.[99] Thus, HIV infection may affect HPV associated neoplasia by both potentiation of the effects of HPV and by leading to immune deficits and defective viral clearing.

Epstein–Barr virus, although demonstrated in invasive squamous carcinoma cells and associated lymphocytes, has not at present been shown to interact with HPV directly.[100]

HORMONES

The URR of HPV 16 contains a glucocorticoid regulatory element, which permits E2 independent early gene transcription.[101] Therefore, steroid hormones may enhance viral transcription, as shown with oestrogens and progestagens in cell lines.[102 103] A recent study has demonstrated that glucocorticoids downregulate HLA class I expression in HPV containing cervical carcinoma cell lines, but only if the viral genome is integrated into the cellular genome.[104] These studies demonstrate that hormonal factors may not only be important in the control of viral gene transcription but that they may also influence immune competence.

IMMUNE STATUS

Certain HLA genotypes are found more frequently in patients with cervical neoplasia than in the normal population,[105–108] suggesting that susceptibility to cervical neoplasia may in part be determined by inherent genetic factors governing the immune response to HPV. Phenotypically, downregulation of class I HLA antigens, which are important in the recognition of viral antigens, is frequent in both preinvasive and invasive disease.[109 110] Conversely, normal cervical epithelial cells do not express class II HLA antigens but such antigens are found on the surface of epithelial cells in cervical neoplasia.[111] This upregulation is related to the morphological grade in preinvasive lesions and appears independent of the presence of HPV sequences.[112] The keratinocyte phenotype in these lesions therefore resembles that of antigen presenting cells and suggests that they may be involved directly in immune surveillance.

In renal transplant patients there is an increased prevalence of wart virus associated changes, CIN, and invasive squamous carcinomas.[113] HIV positive patients also have an increased incidence of these changes which may be associated with both reduced systemic immune competence, as assessed by CD 4 cell count[114] or with a local defect in immunity with reduced Langerhans cell number.[115] This is compounded by local shifts in immune surveillance related to alterations in Langerhans cell activity associated with HPV mediated epithelial cell injury.

In view of the association of viral persistence with lesion progression, variation in the ability of different class II alleles to effect clearance of HPV could explain the association of some HLA class II genotypes with cervical neoplasia. Similarly, acquired immune defects may exert their effects through defective viral clearing and hence viral persistence.

CYTOKINES AND GROWTH FACTORS

In HPV immortalised cell lines E6 and E7 gene transcription can be inhibited by leukoregulin and interferon gamma, but not by interferon alfa, with associated reduction in cell proliferation.[116] HPV 16 and 18 E6 and E7 production is also inhibited by transforming growth factor β (TGF-β) in HPV transformed keratinocytes.[117] Conversely, HPV 16 E5 can interact with epidermal growth factor receptor (EGFR) in the process of cell transformation.[118] E-cadherin transfection leads to restoration of membrane E-cadherin/catenin complex together with downregulation of EGFR and reversion of the transformed phenotype in HPV transfected cells.[119] These studies suggest that interaction of HPV with cell surface molecules may be important in the determination of cellular phenotype.

HPV negative cervical tumours

Sensitive, broad spectrum HPV detection methods have demonstrated HPV DNA sequences in the vast majority of invasive cervical carcinomas. Whether truly HPV negative carcinomas exist is currently a matter for debate as there are several reasons why tumours may falsely appear HPV negative.[120] However, the epidemiological finding that patients with HPV negative CIN have a different spectrum of risk factors suggests that at least intraepithelial disease may arise in the absence of HPV infection.[121] HPV negative cervical cell lines and tumours often contain p53 mutations which could theoretically substitute for the presence of the HPV E6 protein.[122 123] Although there is a substantial inverse correlation between p53 mutation and HPV infection, it has become clear that they are not mutually exclusive in cervical carcinomas. Thus, p53 gene mutations have been identified in HPV positive tumours[124 125] and, similarly, p53 mutations are not always present in apparently HPV negative tumours.[126] This does not however entirely exclude the hypothesis that p53 mutation substitutes for E6 gene expression in HPV negative tumours as (i) p53 mutation may be acquired as a late event in HPV positive tumours[127]; and (ii) apparently HPV negative cervical tumours may be infected with either undetected or unknown HPV types.[120]

Cyclin D is responsible for cells passing through G1 phase and has been implicated in carcinomas when overexpressed.[128] PRAD 1 or *bcl* 1 oncogene, which is on chromosome 11q13 and is activated by translocation, is identical to cyclin D. A recent study has shown abnormalities of PRAD 1 (both amplification and rearrangement of DNA and overexpression of mRNA) in seven of 13 cervical and vulval squamous carcinoma derived cell lines.[129] The E7 protein has cyclin D-like activity and therefore overexpression of cyclin D could mimic, at least in part, the effects of this protein.

Mechanistically, therefore, other genetic abnormalities can substitute for HPV gene expression. This is consistent with the hypothesis that transformation of cervical epithelial cells, and hence carcinoma of the cervix, can occur by HPV independent pathways.

Glandular cervical tumours

The association between glandular neoplasia of the cervix and HPV infection is less strong than that of squamous neoplasia.[130 131] There are methodological difficulties in the study of glandular lesions as, without microdissection techniques, it is not possible to exclude the possibility that any HPV sequences identified in extracted nucleic acids were present in the accompanying squamous epithelium rather than in the glandular lesion.[132] Nevertheless, HPV sequences have been localised directly within glandular epithelial cells by both DNA and RNA in situ hybridisation in several studies.[133-137] Moreover, HPV 18 is found significantly more frequently in glandular than in squamous lesions of the cervix,[131 135 138] indicating the involvement of a different spectrum of HPV types. Small cell carcinoma of the cervix is also associated with HPV 18 infection, suggesting that this HPV type may be involved particularly in non-squamous tumours.[139]

Conclusions

It has become clear that most cervical neoplasia, whether intraepithelial or invasive, is attributable in part to HPV infection. However, HPV infection alone is not sufficient, and may, in a small proportion of cases, not be necessary for full malignant transformation. There is increasing evidence that HPV gene products interfere with cell cycle control leading to secondary accumulation of small scale and large scale genetic abnormalities. This may explain the association of viral persistence with lesion progression but, in many patients, secondary factors, such as smoking and immune response, are clearly important. However, the mechanisms involved in the interaction between HPV and host factors are poorly understood.

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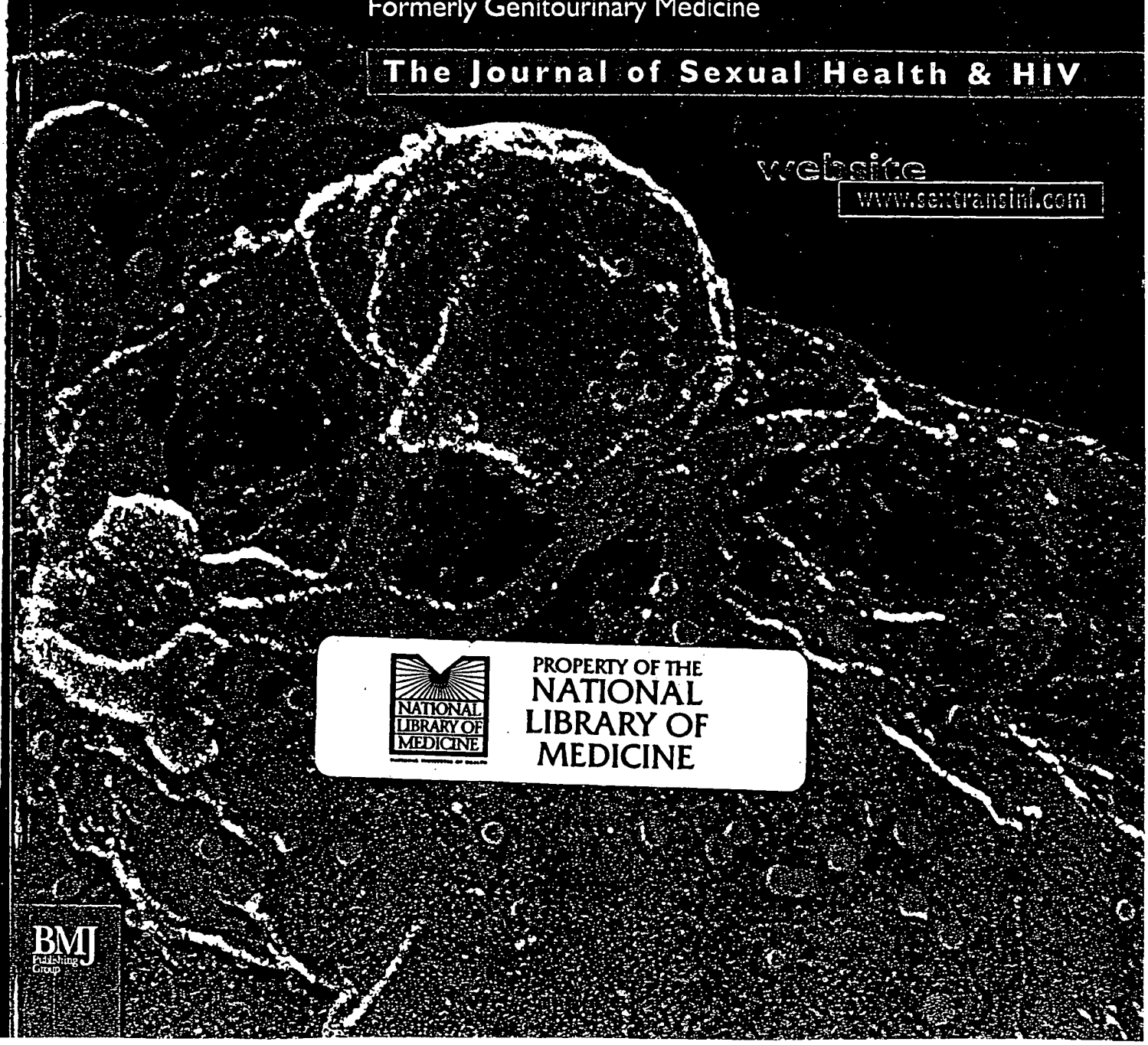

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Human Papillomaviruses and Cervical Neoplasia: A Model for Carcinogenesis

Mark H. Stoler, M.D.

Summary: Human papillomaviruses are etiologic for cervical cancers and their pathologic precursors. As presented in this review, pathologic, epidemiologic, and molecular data all support a working model that accounts for the pathogenetic role of these viruses in cervical neoplasia. Diagnostic criteria and classification systems are discussed in light of this model. These insights point to a potential change in clinical screening systems for cervical cancer. In addition, vaccine trials for oncogenic HPVs have begun. In the long term, these trials may hold promise as truly specific preventive therapy for this common human cancer. Key Words: HPV—Papillomavirus—Cervix—Cancer—Pathogenesis—Screening.

Human papillomaviruses (HPVs) are the etiologic agents of cervical neoplasia. This simple statement is the product of more than two decades of work that has revealed the interplay of these common epitheliotropic viruses with their host cells. The pathologic classification of cervical neoplasia as well as the clinical management of these lesions increasingly reflects these biologic insights. I hope to review these concepts with emphasis on the mechanisms by which HPVs produce abnormal cervical morphology. This will be followed by a brief exploration of some applications of HPV-related technology.

HISTORICAL PERSPECTIVE

Historically, papillomaviruses have co-evolved with vertebrates. Virtually all vertebrate species have warts. Cutaneous warts have been described for thousands of years. In the beginning of this century, Ciuffo established the viral etiology of human warts (papillomas) by using cell-free extracts from wart tissue as an inoculum for man-to-man transmission experiments. In 1933, Shope described the first papillomavirus in cottontail rabbits. Subsequent experimentation in this system, including the use of coal tar as a tumor promoter, stimulated early concepts of cancer initiation and promotion, and produced one of the first examples of a human DNA tumor virus (1–3). The advent of electron microscopy brought the ultrastructural morphology of the papillomaviruses into focus. Clinical studies also revealed that different kinds of warts were more productive of virions than others. For example, plantar warts often had abundant viral particles, whereas genital warts had few (4–7). Since papillomaviruses are resistant to tissue culture and cannot be transmitted to laboratory animals, the characterization of this virus has been extremely difficult. Biochemical characterization and immunology carried out on viral proteins derived from direct extracts of warts provided early data suggesting that there was a single type of human papillomavirus, a view that was held through the 1960s (8). However, in the 1970s the revolution in modern biology permitted the molecular characterization of the papillomavirus family. Clones of the HPV genomes can be used to probe different pathologic processes, to establish the relationship of those lesions with HPVs. Analysis of the genomes isolated from these lesions reveals the plurality of HPV types based on DNA heterology (9,10). Papillomaviruses infect essentially all vertebrate species and induce primarily, albeit not exclusively, squamous epithelial neoplasias. In humans, more than 100 molecular types have been cloned, some two dozen of which are trophic for the anogenital tract (11,12). Anogenital HPV infections are the most com-

From the Robert E. Fechner Laboratory of Surgical Pathology, University of Virginia Health Sciences Center, Charlottesville, VA, U.S.A.
Address correspondence and reprint requests to Dr. Mark H. Stoler, University of Virginia Health Sciences Center, Surgical Pathology, Box 214, Charlottesville, VA 22908.

mon sexually transmitted disease (13,14). The careful correlation of the clinical pathology of HPV-associated lesions with the molecular biology elucidated using the cloned viral DNAs as tools to dissect the virus-host interaction has been the key to our improved understanding of this common human cancer.

HPV VIROLOGY

The papillomaviruses have been traditionally classified as members of the papovavirus family. This family was named by taking the first two letters of the major genera: *pa*pilloma, *po*lyoma, and simian *va*cuolating viruses, respectively. All members of the family have a common structure; they are small, double-stranded DNA viruses that replicate in the nucleus and have icosahedral protein capsules that form nonenveloped virions. However, it has become apparent that the papillomaviruses are biologically distinct from SV40 and polyomavirus. Papillomaviruses have 55-nm rather than 40-nm diameter capsids; a reflection of that fact is that the approximately 8000-bp papillomavirus genome is 60% larger than the genome of polyomavirus. Moreover, the genome of polyomavirus will not cross-hybridize with papillomaviruses under low stringency conditions. The molecular organization of viral transcription is very different between HPV vs. polyoma; papillomaviruses transcribe all of their genes off one strand of the double-stranded genome. In contrast, SV40 and polyoma use a completely different bidirectional transcription strategy.

All papillomaviruses have a circular double-stranded DNA genome of approximately 8.0-kb complexity, encoding seven or eight early and two late genetic open reading frames (ORFs). Through gene splicing, the ORFs encode for all viral gene products. In addition, there is a noncoding region of approximately 1000 bp, often referred to as the upstream regulatory region (URR) or long control region, immediately upstream of the E6 ORF that contains the sequences regulating the expression of all ORFs (15). More than 20 messenger RNAs are expressed, normally in a highly differentiation- and cell-type-specific manner. ORFs E6 and E7 encode proteins that are capable of inducing cell proliferation and transformation. These are the only open reading frames that are conserved and expressed in *all* HPV-associated pathologies. The latter include the full spectrum from low-grade lesions with virtually no neoplastic potential to high-grade invasive cancers. The proteins encoded by E1 are involved in genome maintenance and replication. E2 encodes the major transregulatory proteins, which interact with the URR, having both positive and negative effects on transcription. The E4 ORF is the most abundantly transcribed message in a wart and is most highly expressed in differentiated cells. Some forms of E4 encode a protein, which binds to and disrupts the cytoplasmic keratin network producing what we recognize as a koilocyte, in cells that are appropriately differentiated. E5 also seems to be involved in cell transformation. E5 encodes a small protein that seems to bind to a variety of host membrane proteins including growth factor receptors. It also contains 3′ regulatory and polyadenylation sequences for all of the E region genes. Because expression of E5 is often lost during viral integration, its role in human carcinogenesis is controversial. L2 and L1 encode the minor and major viral capsid proteins, respectively. The expression of these proteins and their messages is also tightly regulated in a cell differentiation-dependent manner.

As noted above, there are more than 100 HPV types. Given the absence of serologic reagents or viral culture systems, these viruses are classified not by serotype, but by genotype. Today, a new HPV type is defined when sequences in selected genomic regions have more than 10% divergence compared to any of the known HPV types. From these definitions and from computerized analysis of these sequences, it is clear that the papillomaviruses have had a long evolution, probably coevolving with humans as well as vertebrates in general (16–19). The different viral types are not the product of simple point mutation. The grouping of papillomaviruses that is derived from sequence analysis remarkably predicts the recognized clinical groupings (20). Broadly speaking, there are cutaneous and mucosotropic groups. In the cutaneous group, there are HPVs common to the general popualtion, such as HPV-1, which is the agent of plantar warts, HPV-2 and -4, which cause common warts, and then there is a large group of 20 or more HPV types that are associated with the rare disease epidermodysplasia verruciformis (EV). Interestingly, most of the EV warts that progress to cancer are associated with HPV-5 and -8, i.e., there is a "high-risk" subgroup analogous to the subgroups recognized in the mucosotropic HPVs. In the mucosotropic group, the viruses may be broadly classified into those with a low risk of lesion progression to cancer vs. those with a moderate-to-high risk. Viruses classified as low risk are defined by the fact that they are almost never found in invasive cancers. In contrast, high-risk viruses are those that are most often found in invasive cancer. However, high-risk virus infection does not equate to the inevitable development of cancer. The molecular epidemiology of most of the moderate- or intermediate-risk viruses seems incompletely developed because of a relative lack of probes and the recent description of members of this group.

The four mucosotropic viruses, HPV-6, -11, -16, and -18, form the prototypes for the low- vs. high-risk groupings and together account for approximately two-thirds of the HPV-associated anogenital neoplasms (21). Type 6 and 11 primarily cause benign exophytic genital warts or condylomata acuminata. These are the viruses present in more than 90% of condylomas with about two-thirds caused by HPV-6 and one-third by HPV-11. They are also associated with low-grade squamous intraepithelial lesions (LSIL) and are only rarely associated with high-grade squamous intraepithelial lesions (HSIL) or invasive squamous cancers. Related viruses that produce a similar spectrum in the cervix are HPV-42, -43, -44, as well as -26, -53, -54, -55, -62 and -66. In contrast, HPV-16 is the most prevalent virus to infect the uterine cervix, is closely associated with the entire range of intraepithelial and invasive squamous neoplasia, as well as less commonly, cervical glandular neoplasia. The moderate-to-high risk types most closely related to HPV-16 include types -31, -33, -35, -52, -58, and -67. HPV-18 is the other cancer-associated prototype, which is also most commonly associated with nonsquamous cervical neoplasms. The viruses most closely related to type 18 include types 45, 59, as well as types 39 and 68. Other types such as 51 and 56 seem to have some association with cervical cancer but are genetically also related to the cutaneous group. Morphologically similar lesions at different mucosal sites are largely caused by the same mucosal viruses. Thus, laryngeal and conjunctival papillomas, which are pathologically and biologically equivalent to a condyloma, are most often caused by HPV-11 and -6. In contrast, the bowenoid dysplasias of the vulva, penis, anus, and oral cavity are most often associated with HPV-16. As will become clear, all HPV types, even the high-risk viruses, must induce the pathologic equivalent of a wart, condyloma, or LSIL, for this is pathology that supports viral replication and virion production.

HPV EPIDEMIOLOGY

Numerous epidemiologic studies have linked cervical cancer to sexual behavior (13,14,22–26). In most studies, the finding that either female promiscuity or being a monogamous female partner of a promiscuous husband confers increased risk for cervical cancer supports the concept of a sexually transmitted agent. The strongest epidemiologic risk factor is the number of sexual partners. Other behavioral correlates such as young age at first intercourse and early age of parity also seem to confer risk, although the altered hormonal environment of adolescence and its effect on the cervical epithelium may also be a risk factor. In most epidemiologic studies, cigarette smoking remains a risk factor even after controlling for sexual factors. Morphoepidemiologic studies demonstrate that the precursors of cervical cancer precede invasive cancer, with LSIL having the highest prevalence in patients in their early twenties, HSIL in the late twenties and early thirties, and invasive cancer in women ages 40 to 50. In cohorts that are more recent, there is a trend toward earlier age for each of these stages.

The epidemiology of genital HPV infection clearly accounts for the epidemiology of cervical neoplasia (24,26). Modern molecular epidemiologic analyses, as well as our understanding of the molecular biology of the virus-host cell interaction, provide a mechanistic basis for this link. The confusion in the literature of the 1980s that failed to show a strong association between HPV and cervical neoplasia was most likely due to a lack of high-quality molecular tests for HPV DNA. More recent analyses using validated methods confer relative risks for cervical neoplasia based on the presence of HPV on the order of 10 to 100 to one. These are at least an order of magnitude greater than any other epidemiologic risk factor for cancer ever described. In multivariate analyses, controlling for the presence of HPV infection leads to "dropout" of virtually all other risk factors. Thus, HPV infection confers 85 to 90% of the attributable risk for the development of cervical dysplasia. Cohort studies have shown that in cytologically normal women HPV infection precedes the development of dysplasias and that infection with HPV-16 or -18 confers the highest risk for dysplasia, particularly high-grade dysplasia. For example, in a study of 241 cytologically normal women recruited in a sexually transmitted disease clinic, the cumulative incidence of HSIL at 2 years was 28% in HPV-positive women compared to 3% in HPV-negative women (27). In a study of more than 200 "atypical" Pap smears that were reread by five expert cytopathologists, it was shown that the HPV prevalence increased from 21% in smears classified as normal to 100% in smears unanimously classified as SIL (28).

The prevalence of HPV in a population varies with the population and the method of HPV detection (29). Tests using highly sensitive amplification methods capable of detecting many viral types demonstrated the highest prevalence. When a cohort of college women were studied using L1 consensus primer polymerase chain reaction (PCR) in parallel with the commercially available Vira-Pap dot blots which only had probes for seven viral types, the HPV prevalence was 46% for the PCR assay vs. 11% for the dot blot (30,31). A conservative estimate of genital HPV prevalence in the general U.S. population is probably on the order of 15 to 20%, with serial sam-

pling over time leading to even higher estimates of prevalence. However, these rates vary with age, suggesting that patients may also clear their infection over time (32,33). Of course, this parallels cytologic observations that most cervical abnormalities regress, with only rare cases progressing to HSIL and even more rarely to invasive cancer (34–39). The overwhelming clinical problem is trying to decide which cases of low-grade morphology will or will not progress so as to more effectively direct therapy to those who need it.

HPV PATHOLOGY

The pathology of papillomavirus-associated neoplasia describes the neoplastic pathology of the cervix. In 1956, Koss coined the term koilocytotic atypia (KA) to insightfully describe cells derived from flat "warty lesions" of the cervix (40). KA is often thought to be pathognomonic for HPV infections, i.e., HPV cytopathic effect. It is the cytologic abnormality in which virion is most often detected, and is highly correlated with productive HPV infection. However, cytologic or histologic absence of KA does not in any way imply absence of HPV, more specifically absence of pathogenic HPV gene expression. Koilocytotic atypia, which under the Bethesda classification is considered a low-grade squamous intraepithelial lesion, is the most common definite abnormality in cytologically screened populations today, present in 1 to 4% of Papanicolaou ("Pap") smears (41,42). In 1976, Canadian and Scandinavian workers both described flat and inverted condylomas of the uterine cervix and reported that these lesions not only may be found in the spectrum of cervical intraepithelial neoplasia (CIN), but that they also were associated with the human papillomavirus (43–45). Supporting morphologic data linking HPV to cervical neoplasms came initially from studies of the distribution of HPV capsid protein or HPV virions in intraepithelial neoplasias. Using a broadly reactive group-specific antisera against the L1 protein, papillomavirus capsid antigen, the expression of which is a highly differentiation-dependent phenomenon, was found to be present in 50 to 60% of condylomas or LSILs with a decreasing frequency as cytohistologic grade increased (46–48). Similarly, if one uses transmission electron microscopy to search cell nuclei for virions, there is an inverse correlation with cytologic lesion grade for the detection of virus (49–51).

HPV MOLECULAR BIOLOGY

Data derived from molecular biologic studies of HPVs support the epidemiologic and pathologic associations. Molecular detection methods can be applied in a variety of ways. Analyses that destroy the sample to release the nucleic acids for analysis by necessity require morphologic correlation. Most commonly, a cellular sample is analyzed for the presence of HPV DNA by dot blot, Southern blot, or some analogous technique, more recently with improved sensitivity by coupling these methods to an amplification technology like the PCR (20,52–54). These types of studies have been strongly complemented by many direct analyses, which have used *in situ* hybridization to directly demonstrate the presence of HPV DNA or messenger RNA in defined groups of pathologies (55,56).

There are more than two dozen mucotropic HPVs that infect the genital tract. All can be found in low-grade SILs or in samples from cytologically "normal" women, and although no single type predominates, the HPV-16-related groups are the most common viruses in the cervix (20,57). The prevalence of HPV DNA in LSILs is in excess of 90%, and the same may be said for HSIL and invasive squamous carcinoma of the cervix. However, the type spectrum in the high-grade lesions is much more restricted, with just four HPV types (16, 18, 31, and 45) accounting for almost 80% of the invasive cancers. Squamous cell carcinomas account for only 80% of cervix cancers, the remainder being made up of primarily endocervical adenocarcinomas and a small number of small cell neuroendocrine carcinoma (58). Studies using sensitive methods to analyze these nonsquamous cancers and their precursors also demonstrate a very high prevalence of HPV DNA (59–62). The virus most closely related to progressive cervical squamous neoplasia is HPV-16 (63). Although accounting for fewer cervical infections, HPV-18 is more consistently associated with adenocarcinomas and small cell neuroendocrine cancers of the cervix and less frequently with invasive squamous cancer. The absolute prevalence of some of the more recently described types (e.g., HPV-31, -33, -35, -39, -42, -43, -44, -45, -51, -52, -56, and others) may be underestimated, as they have not been generally available for large-scale screening (64). Thus, the best available evidence suggests that HPV genetic material is present in more than 90% of premalignant and malignant squamous lesions of the uterine cervix. A corollary of this is that studies reporting lower association rates may be technically deficient and have a significant false negative rate (*vida infra*). The association of HPVs with squamous cell carcinomas at other body sites (vagina, vulva, anus, penis, larynx, and skin) and in a variety of genetic or induced immunodeficiency states is also well recognized (65–68).

Unlike any other candidate cervical cancer agent, HPV

DNA is not only present in every pathology linked to HPV, but also HPV messenger RNA is expressed in these lesions (69,70). The presence of viral RNA and protein expression leads to a rational framework implicating the virus in lesion pathogenesis. Patterns of viral mRNA expression vary with morphology in a tightly regulated and differentiation-dependent manner (55,69,71,72). In low-grade lesions, all viral genes are expressed as a manifestation of vegetative viral replication. In contrast, in HSIL and invasive cancer, there is a restricted pattern of viral gene expression, and E6 and E7 predominate. Cervical carcinoma cell lines such as HeLa, SiHa, and CaSki have also been found to harbor integrated HPV-16 or -18 DNAs from which the transforming E6 and E7 regions are actively transcribed (73–78).

Active transcription of HPV DNA within lesions establishes a strong molecular association of HPV with cervical neoplasia. *In vitro* cell transformation experiments additionally point to an active role for these viruses in carcinogenesis. DNA from high-risk HPV types like HPV-16, -18, -31, and -33, but intriguingly, not HPV-6 or -11, are capable of transforming epithelial cell lines in cooperation with an activated cellular oncogene such as Ha-ras, thus mirroring general concepts of multistep carcinogenesis (79–83). HPV-16 DNA alone can immortalize cultured primary foreskin keratinocytes or primary cervical cells in culture (84–87). While not inhibiting stratification of keratinocytes cultured on collagen rafts, HPV-16 can prevent cellular differentiation, thereby inducing in these artificial epithelia morphologic features that mimic CIN (88,89). Deletion experiments clarified that the essential part of the viral genome for these effects is the expression of the E6 and/or E7 region. It is also noteworthy that in these systems the transformed phenotype is not apparent until the cells have been passed through many generations, mimicking the long progression times seen in naturally occurring clinical lesions and suggesting the need for additional genetic events to manifest a high-grade lesion.

There is also an association between the physical state of HPV DNA within the cell and the malignant potential of the associated epithelial proliferation (90–95). In benign HPV-infected lesions, the viral DNAs exist as extrachromasomal plasmids, mostly as monomeric circular molecules (96). However, in most cancers, HPV DNAs are integrated into host chromosomes. Viral integration most frequently disrupts the E2 ORF, which encodes the transcription regulatory proteins. Loss of these regulatory proteins is thought to be the basis for potential dysregulation of the expression of the transforming E6 and E7 ORFs (97).

Concurrent with the revelation of HPV biology, there has been an explosion of information about the roles of cellular oncogenes in carcinogenesis (98–101). Several classes of oncogenes, including growth factors, growth factor receptors, GTP binding proteins, protein kinases, and DNA binding proteins, have been shown to be relevant to the control of cell growth. C-myc and c-Ha-ras amplification can be documented in some cervical cancers and correlates with advanced clinical stage at the time of analysis (102–105). In cervical cancer cell lines, HPV integration sites were found to be in the same general region as some of the known oncogenes, including c-myc, suggesting the possibility of transcriptional activation by the virus, although the latter has not been directly documented (106,107). In other cases, HPV DNA integrates near fragile sites (108). The significance of these observations is not clear, but again suggests the potential for multiple genetic/chromosomal events in neoplastic progression.

Observations on oncogene effects have been hard to directly relate to pathogenesis. In contrast, elucidation of the interaction of HPVs with tumor suppresser genes has been highly informative. Fusion of HPV-18-expressing HeLa cells with normal human fibroblasts or keratinocytes results in the repression of the malignant phenotype of the HeLa cell (109). Upon transplantation into nude mice, the loss of chromosome 11 from the hybrid cells results in the reversion to malignant phenotype, suggesting another tumor suppressor gene at this site. This experiment was extended by Schwarz and coworkers, who proposed that the ability of a cellular product to suppress the expression of the HPV-18 oncogene requires a humoral factor (68,110,111). Clearly, several gene products may interact to elicit or inhibit cell transformation. The human retinoblastoma (RB) gene was the first tumor suppressor gene to be characterized (112). RB either is completely absent or has significant deletions in tumors from patients with retinoblastoma, breast cancer, and in several other epithelial tumors such as squamous cell carcinoma of the head and neck (113–116). The transforming E7 protein of HPV-16 has structural and functional similarities to the E1A antigen of adenovirus, the large T antigen of SV40, and the host cellular protein cyclin D1 (117–122). All of these proteins have the ability to form inactivating complexes with the retinoblastoma antioncoprotein by competitive binding to the "RB pocket." This functional inactivation causes the release of a potent host transcription factor, termed E2F, which is capable of activating transcription of a variety of host genes, many of which are involved in DNA synthesis and cell cycle progression. Similar complexing and inactivation of the p53 suppressor gene by the E6 proteins of

high-risk viruses like HPV-16 has also been demonstrated (123–129). E6 binds to p53 via an E6-associated host protein. This binding promotes the ubiquitin-dependent degradation of p53, the functional equivalent of mutational inactivation. p53 is a prime regulator of cell proliferation via transcriptional transactivation. For instance, p53 activates transcription of p21 (also called waf 1 or cip 1), a potent inhibitor of cyclin-dependent kinase. Therefore, either mutation or E6-mediated degradation of p53 can lead to derepression of cell cycle regulation. In rare instances in which a cervical cancer has been shown to not contain HPV, p53 mutation has been found, whereas mutation is absent in the usual case (130–133). Interestingly, the E6 proteins from low-risk HPVs are incapable of causing this degradation. Therefore, E6 undoubtedly has other roles in the virus-host interaction other than p53 inactivation, such as direct effects as a transcription factor (134).

HPV-MEDIATED CARCINOGENESIS

Taken together, the above data have led to a molecular model for HPV-induced carcinogenesis. This model involves the interaction of HPV gene products with what is recognized to be a tightly controlled network of cellular oncogenes and antioncogenes, which control cell proliferation and DNA synthesis. Histogenetically, papillomaviruses must infect the "reserve, basal, or stem" cell population of the cervical transformation zone, cells that have the potential to differentiate along squamous, glandular, or neuroendocrine lines and are responsible for epithelial maintenance. In cells committed to squamous differentiation, there is an orderly program of maturation throughout the epithelial thickness, both at the morphologic and molecular level. In normal squamous differentiation, the only cells capable of cell division are the basal or parabasal cells. In morphologically normal, but HPV infected, basal cells, papillomavirus gene expression is inhibited to essentially maintenance levels. Productive HPV gene expression is tightly regulated and permitted only in cells that have begun squamous maturation, with concurrent loss of proliferative capacity (55,71,72,135–138). In the immediate suprabasal zone, there is expression of the early regions of the virus, and as the cells differentiate, there is an induction of all viral genes, as well as viral DNA synthesis, leading to assembly and production virions in the cells just beneath the surface. In the cervix, we recognize such lesions as low-grade squamous intraepithelial lesions or mild dysplasias, most of which at some point demonstrate koilocytotic atypia. Such lesions usually regress or maintain themselves for extended periods. An explanation of some of the diagnostic criteria used by pathologists is implicit in this program of differentiation-linked expression. The nuclear enlargement and hyperchromasia recognized as atypia is a direct result of E6/E7-mediated activation of host DNA synthesis. In a low-grade lesion, this is regulated to occur in cells that can no longer divide, i.e., the intermediate squamous cells, and is primarily directed at the production of viral DNA (138). Given the small size of the viral genome, the several thousand copies of the virus present in a productively infected cell clearly cannot account for the two- to fourfold nuclear enlargement that is observed. It is a diagnostically fortunate coincidence that ineffective (in the sense of cell division) E6/E7-mediated host DNA synthesis produces the enlarged nuclei and increased N:C ratio that one recognizes as abnormal. If the process is not fully developed or is perhaps regressing, then the cells derived from the surface often have less nuclear abnormality (? atypical squamous cells of uncertain significance [ASCUS]) than seen in classical dysplasia. In contrast, in the fully developed case, they are classified as being derived from a mild dysplasia/LSIL. If the cells also have the correct amount and form of the cytokeratin-binding protein HPV E4 expressed, then they appear as koilocytes. Koilocytotic atypia, while very often present, does not have to be seen to recognize a low-grade lesion. Every cytologist recognizes cells derived from the upper levels of a mild dysplasia that meet the diagnostic criteria for dysplasia, yet do not have the characteristic perinuclear halo termed koilocytosis. Such lesions are just as HPV-associated as those that do have koilocytes, and the differences undoubtedly represent temporal variation within the life cycle of a low-grade lesion.

If viral gene expression is so tightly regulated, how do high-grade lesions develop? The *sine qua non* of high-grade dysplasia is morphologic evidence of basal-like cell proliferation. In these cells, the coordinate link between differentiation and viral early gene expression is lost. How this occurs is unclear, although it certainly must be a rare event(s) given the relative frequency of low vs. high-grade lesions. Potential mechanisms might include viral integration or mutations in HPV E2, such that E2-controlled regulation of E6/E7 expression is lost. In such cases, the viral oncogenes E6 and E7 are inappropriately expressed in a population of cells that retain the capacity to divide, thereby initiating cell proliferation. As this population of cells proliferates, it overtakes the epithelium, producing lesions that are by definition characterized by less orderly squamous maturation and basal-like cell overgrowth. Possible promoters of this process could be smoking, other viruses, random mutation, etc. The relative infrequency of these effects is bio-

logically manifest by the latency and relative rarity of HSILs vs. LSILs. Progression to the proliferative phenotype occurs most frequently, albeit not exclusively, with high-risk viral types, and results in the high-grade squamous intraepithelial lesions also called moderate squamous dysplasia, severe squamous dysplasia, or squamous carcinoma *in situ*. Thus, the Bethesda system's break between low-grade vs. high-grade follows in part from the biologic changes manifest between these morphologies. Indeed, from the standpoint of epithelial biology, there is little rationale for separating moderate from severe dysplasia in that the critical break occurs between mild and moderate dysplasia, with the switch to a proliferative as opposed to a differentiated phenotype.

In high-grade squamous intraepithelial lesions, the proliferating basaloid cells, driven by E6/E7 overexpression, are at much greater risk for the acquisition of additional genetic errors, clonal selection, etc., perhaps under the influence of the same external mutagens and/or host genetic predisposition, which further promotes the development of the fully malignant phenotype, most often an invasive squamous cell carcinoma. The different subtypes of squamous cancer are probably related to the multistep and somewhat random nature of the process. The proportion of different types just reflects the relative likelihood of different genetic pathways to a "successful" cancer, in part modulated by the microenvironment in which the lesion develops. Hence, early observations that keratinizing cancers are often more ectocervical than large cell-nonkeratinizing or small cell malignancies, which tend to originate higher in the endocervical canal, have some contemporary validation.

Given this model for cervical squamous neoplasia, one still needs to account for glandular and small cell neuroendocrine neoplasms. Of course, reserve cells that are already committed to glandular differentiation are, because of a lack of an appropriate differentiation environment, not going to be productive of virions. The productive viral life cycle requires the cellular milieu of orderly squamous differentiation. If this is true, then viral infection in cells committed to glandular differentiation most often results (from the viral standpoint) in an abortive or latent infection of morphologically normal endocervical cells. Rarely, dysregulation of viral early gene expression occurs in these usually nonpermissive cells. This leads to hyperproliferative lesions of glandular cells, which pathologists recognize as severe endocervical dysplasia/adenocarcinoma *in situ* (AIS). There is no biologic correlate in this model of a low-grade glandular dysplasia. Hence, this explains the inability of pathologists to reproducibly recognize, either cytologically or histologically, a clinically meaningful lesion less severe than what most call AIS. HPV-18 seems to be more successful at inducing this in glandular cells than HPV-16. Perhaps this is because HPV-18 has a greater disposition to integrate into the genome and perhaps because it may have some preference for cells predisposed to other than squamous differentiation. Parenthetically, little if anything is known about the mechanism of HPV-type-specific cellular tropism. However, no HPV type can be exclusively trophic for nonsquamous cells, because if this were so, that strain of virus would be eliminated from the population. Depending on the genetic switches that over time accompany virally induced glandular proliferations, the outcome may be an invasive adenocarcinoma, most often endocervical, but less frequently of another type, e.g., endometrioid, clear cell, etc. The relative frequencies of the different types of cervical adenocarcinomas again may just reflect the relative frequency of the different populations committed toward various types of differentiation. Essentially identical arguments may be made for the development of small cell neuroendocrine carcinomas, tumors that are almost always associated with HPV-18 and whose low incidence probably reflects the relative abundance of a susceptible neuroendocrine-committed precursor cell population and the rarity of "successful" viral induction of cell proliferation in such cells. None of the above precludes alternative pathways of carcinogenesis unrelated to HPV (105,138,139). However, in the uterine cervix, the ubiquity of HPV infection is the predominant force driving neoplastic development. Fortunately, when compared to the high prevalence of the virus, progression is an extremely rare occurrence.

HPV SCREENING

As noted earlier, the estimated overall HPV prevalence in the U.S. target population is approximately 20%. The prevalence varies greatly with age. For female ages 20 to 29, the prevalence is probably 40 to 50%, and this decreases by 50% for each decade of age until a background level of around 5% is reached. These data have implications for a brief discussion about the utility of human papillomavirus testing as a screening procedure.

By now it should be clear that virtually all lesions encompassed by the term "cervical neoplasia" are HPV-associated. The epidemiologic and molecular evidence supporting this finding has been presented and is hopefully convincing. Furthermore, virtually 100% of invasive carcinomas from around the world have been shown to be associated with a limited spectrum of HPV types (135,140–142).

Given the strength of these associations, an obvious question is whether screening for HPV using some sort

of molecular diagnostic would be superior for selecting the population at risk for cancer development (143–147). The answer to this apparently simple question is unfortunately complex. Part of the problem is technical. Which HPV test should be used? HPV testing, as all molecular diagnostics, is continually evolving (144,145,148–152). Until recently, there has been only one commercially available FDA approved test for HPVs, the Hybrid Capture Tube test (HCT) marketed by Digene Diagnostics (Gaithersburg, MD). The sensitivity, specificity, and predictive values of the "tube" test with a 10 pg/ml cutoff value for a group of 11 to 13 high-risk viruses have been well-characterized (152–154). Compared to PCR analysis using L1 consensus primers, the HCT has a lower sensitivity. However, the HCT is more specific for the presence of clinically detectable cervical abnormalities compared with PCR, which because of its higher sensitivity, picks up a significantly higher population of patients without clinically detectable disease.

As noted above, molecular technologies continue to evolve. The newest version of the Hybrid Capture test (HC II, approved in March 1999) is relatively semiautomated, uses a microtiter format, and has up to 50 times the analytic sensitivity of the current test. Whether the improved sensitivity is of clinical benefit greatly depends on whether one is using the test for screening vs. diagnosis/triage and the population characteristics. The interplay between sensitivity, specificity, and disease prevalence needs to be considered when evaluating the utility of any test. Likewise, PCR/amplification technologies are rapidly evolving. In addition, the expanding sequence database of all relevant human papillomaviruses makes it likely that the new powerful "DNA-chip" technologies may possibly replace or augment current HPV testing methods.

Might HPV testing be a better screening method? This question has been most thoroughly examined by workers in the Netherlands who have proposed using an extremely sensitive PCR-based method as the first step in a cervical cancer screening program (155–157). If one were designing a cervical cancer screening program from scratch, this approach makes a tremendous amount of sense. Nearly 100% of the pathology of interest is HPV-positive. Conversely, if after using a sufficiently sensitive screening test an individual were not HPV-positive, the incidence of disease would be so low as to make screening nearly worthless. Combining the high prevalence of human papillomaviruses in the pathology of interest with the relatively long time frame from acquisition of infection until the development of the target, cervical cancer, immediately brings the relative value of initial triage based on HPV status into focus. The lower the prevalence of HPV in the population to be screened, the better the performance profile of an extremely sensitive HPV screening test. For instance, the incidence of cervical cancer in women under 25 to 30 years of age is extremely low, and the prevalence of HPV in the United States drops from approximately 40% at age 20 to 10 to 20% at ages 30 to 40 (or as low as 4 or 5% at age 30, as it is in the Netherlands). Under these conditions, it may not make sense to spend resources on screening young women, most of whom develop only transient, low-grade lesions. The Dutch proposal seeks to screen the entire population at age 30 with the most sensitive available HPV test, combined with a single cytologic screening. Patients who are positive on either test would be entered into a program of more intense routine screening, whereas the "double negative" patients would be returned to the general population pool that would be then screened on the long-interval basis of 5 to 10 years. Again, if the prevalence of detectable virus is low and the disease prevalence is also low, such a system makes for extremely rational triage and resource utilization. The arguments become even stronger if the cost and reliability of the HPV test becomes comparable to cytologic methods. Indeed, in some recent studies, HPV testing seems more reliable than the Pap smear due to superior sensitivity in identifying patients with cervical abnormalities. For instance, a large, recently published triage study of ASCUS patients evaluated Hybrid Capture II testing for "oncogenic" HPVs vs. repeat smear as an index for colposcopic referral (158). The sensitivity for HSIL+ in the HPV testing arm was 89.2% with a specificity of 64.1%. In contrast, the sensitivity for repeat Pap smear was 76.2%. This difference approached statistical significance. This and other studies strongly suggest that HPV testing will evolve into routine clinical practice. Furthermore, prospective studies addressing a rational basis for HPV primary screening are needed and planned in the Netherlands and at other sites. Whether such a program could be tested in the United States is debatable given the relative mobility of the U.S. population and the strongly ingrained emphasis on annual Pap smear screening.

HPV VACCINES

The recognition that human papillomaviruses are the primary etiologic agent for cervical cancer strongly raises the possibility of the use of HPV vaccines both for the potential treatment as well as prophylaxis of cervical cancer (23,159–167). A successful prophylactic HPV vaccine could virtually eliminate the need for cervical cancer screening programs. This admirable long-term goal is just possibly coming into reach.

The multiplicity of viral types in the cervix is a problem for vaccine development. It is unclear whether immunity to any specific type provides cross-reactive immunity to other types. Thus, the ultimate vaccine may likely be a complex polyvalent mixture. Until recently, the lack of an abundant source of HPV antigens has markedly impeded vaccine development. However, recombinant methods capable of generating virus-like particles containing the HPV L1 and L2 capsid proteins have been the major technical advance promoting HPV vaccine development. Studies performed in animals reveal consistent and promising findings for the development of a prophylactic HPV vaccine. Vaccines developed in rabbits, cows, and dogs all show great promise. Canine oral papillomaviruses (COPVs) are effectively prevented by intradermal injection in the footpad of either a formalin-inactivated COPV wart extract or COPV L1 virus-like particles. Immunization of approximately 60,000 beagles over a 3-year period resulted in complete protection against naturally acquired COPV-induced warts.

Approximately 80% of HPV cancers are associated with a limited type spectrum of HPV-16, -18, -31, and -45. Several vaccine trials, most initially targeting HPV-16, are undergoing Phase I and Phase II testing. Obviously, the long natural history of both HPV infection and cervical cancer, together with the fact that the optimal target population involves young people, before the onset of sexual activity, complicates the development of such vaccines. However, the potential success of an HPV vaccine program could produce the first example of true cancer prophylaxis, and ultimately lead to the elimination of the entire cervical cytology screening system.

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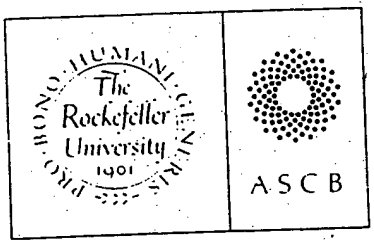

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Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line

Petra Boukamp, Rule T. Petrussevska, Dirk Breitkreutz, Jürgen Hornung, Alex Markham,* and Norbert E. Fusenig

Division of Differentiation and Carcinogenesis in Vitro, Institute of Biochemistry, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany; and *ICI Diagnostics, Gadbrook Park, Rudheath, Northwich Cheshire, England

Abstract. In contrast to mouse epidermal cells, human skin keratinocytes are rather resistant to transformation in vitro. Immortalization has been achieved by SV40 but has resulted in cell lines with altered differentiation. We have established a spontaneously transformed human epithelial cell line from adult skin, which maintains full epidermal differentiation capacity. This HaCaT cell line is obviously immortal (>140 passages), has a transformed phenotype in vitro (clonogenic on plastic and in agar) but remains nontumorigenic. Despite the altered and unlimited growth potential, HaCaT cells, similar to normal keratinocytes, reform an orderly structured and differentiated epidermal tissue when transplanted onto nude mice. Differentiation-specific keratins (Nos. 1 and 10) and other markers (involucrin and filaggrin) are expressed and regularly located. Thus, HaCaT is the first permanent epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for studying regulation of keratinization in human cells. On karyotyping this line is aneuploid (initially hypodiploid) with unique stable marker chromosomes indicating monoclonal origin. The identity of the HaCaT line with the tissue of origin was proven by DNA fingerprinting using hypervariable minisatellite probes. This is the first demonstration that the DNA fingerprint pattern is unaffected by long-term cultivation, transformation, and multiple chromosomal alterations, thereby offering a unique possibility for unequivocal identification of human cell lines.

The characteristics of the HaCaT cell line clearly document that spontaneous transformation of human adult keratinocytes can occur in vitro and is associated with sequential chromosomal alterations, though not obligatorily linked to major defects in differentiation.

CELLS from several normal tissues of rodent origin can be malignantly transformed in vitro by various agents and can also undergo spontaneous neoplastic conversion after variable periods in culture (Sanford and Evans, 1982). Human cells have been found to be rather resistant in this respect (DiPaolo, 1983; for further references see Barrett and Tennant, 1985). The differences between human and rodent cells in their sensitivity to transforming agents in vitro have been attributed to several discriminating species-related factors such as natural life span, degree of inbreeding, and genetic stability. Since the incidence of neoplastic transformation in different species appears to correlate with the frequency of spontaneous chromosomal aberrations, the low incidence of complete transformation of human cells in culture has been mainly ascribed to the higher stability of the human genome (Sager et al., 1983; Kano and Little, 1985).

To date, transformation of human keratinocytes has only been achieved reproducibly by infection with simian virus-40 (SV40) or transfection with its isolated DNA (Steinberg and Defendi, 1979; Taylor-Papadimitriou et al., 1982; Banks-Schlegel and Howley, 1983; Brown and Parkinson, 1984; Rhim et al., 1985) resulting in potentially immortalized but nontumorigenic cell lines. These cells exhibited altered growth properties and substantial reduction of normal keratinization as well as partial reexpression of fetal characteristics (Banks-Schlegel and Howley, 1983; Steinberg and Defendi, 1983; Bernard et al., 1985). From these findings, as well as those from skin carcinoma cell lines (Rheinwald and Beckett, 1980; Tilgen et al., 1983; Boukamp et al., 1985) and from human tracheal and skin epithelial cell lines malignantly transformed in vitro using viruses or viral genes (Rhim et al., 1985; Yoakum et al., 1985), it has been concluded that deficiencies in differentiation are invariably associated with transformation and/or malignancy.

In contrast, we have demonstrated with spontaneously transformed mouse cells that immortality as well as malignancy can be compatible with a largely preserved differentiation potential and sensitivity to appropriate environmental signals (Breitkreutz et al., 1986; Fusenig et al., 1985; Bohnert et al., 1986). To explore whether this is also the case for transformed human keratinocytes, and to exclude virus-related effects on differentiation, we attempted to promote

spontaneous transformation in human skin keratinocytes by a variety of different culture conditions. Spontaneous transformation in vitro, including malignant progression of human cells, has been observed only rarely, even when cells were derived from cancer-prone patients (Azzarone et al., 1976; Danes and Sutano, 1982; Mukherji et al., 1984; Thielmann et al., 1983; Revoltella et al., 1986). During preparation of this paper another case of spontaneous transformation of human keratinocytes has been published (Baden et al., 1987*a*) using neonatal foreskin cells supposedly derived from a genetically affected individual.

Various methods for the propagation of human epidermal cells in vitro have been reported in recent years, mainly using irradiated 3T3 cells as feeder layers (Rheinwald and Green, 1975) or other modified culture conditions (e.g., Eisinger et al., 1979; Peehl and Ham, 1980; for review see Fusenig, 1986). The forced stimulation of proliferation achieved by these conditions and the presence of aneuploid transformed heterologous cells might not be optimal for transformation studies. Our experiments with mouse keratinocyte cultures have shown that spontaneous transformation to immortality and finally malignancy was best and most reproducibly achieved under conditions where cells could be successfully maintained in primary culture for long-term periods (Fusenig et al., 1982, 1985).

Using this approach we describe herein the spontaneous development of a cell line (HaCaT) from a long-term primary culture of human adult skin keratinocytes. These cells can be considered immortal (>140 passages), reveal a heteroploid stemline with specific stable marker chromosomes, but are not tumorigenic. Even after multiple passages HaCaT cells retain a remarkable capacity for normal differentiation and thus offer a suitable and stable model for keratinization studies. Moreover, this line could be reproducibly transfected with the activated human Ha-ras oncogene. Selected clones gave rise to highly differentiated benign epidermal cysts and/or squamous cell carcinomas in nude mice (Fusenig et al., 1987; Boukamp, P., D. Breitkreutz, E. Stanbridge, P. Cerutti, and N. E. Fusenig, manuscript in preparation).

Materials and Methods

Cell Isolation and Cultivation

Full thickness adult human body skin was obtained by surgical excision, in the case of HaCaT from the distant periphery of a melanoma located on the upper half of the back (not extensively sun-exposed) of a 62-yr-old male patient. The histology of the epidermis from the skin specimen obtained in a second, "safety" operation at the primary melanoma site showed no apparent anomalies. For cell isolation, skin was freed from fat and as much dermis as possible, cut into 1 cm^2 pieces, and placed on a 0.2% trypsin solution (1:250; Boehringer Mannheim, Mannheim, Federal Republic of Germany) in PBS without Mg^{2+} and Ca^{2+} at 4°C for 24–72 h depending on the thickness of the skin. Epidermis and dermis were then separated and cells isolated from both parts since the split level varied with different specimens (never located only at the basement membrane). Both cell fractions released from epidermis and dermis were pooled in complete culture medium of 4× MEM (a modified MEM, containing a fourfold concentration of aminoacids and vitamins, plus 15% heat-inactivated FCS and antibiotics; Fusenig and Worst, 1975), filtered through nylon gauze (100-μm mesh width), centrifuged, resuspended in complete culture medium, counted (Coulter counter; Coulter Electronics, Krefeld, FRG) and seeded at a density of 1.5 × 10^6 cells per 30-mm plastic petri dish (Falcon Labware, Oxnard CA) in medium containing ~1.4 mM Ca^{2+} (high Ca^{2+} 4× MEM). When islands had formed (by 5 d after plating) the Ca^{2+} concentration was reduced to ~0.2 mM (low Ca^{2+} 4× MEM) by using Ca^{2+}-free 4× MEM, 5% FCS, and 5% chelex-treated (Ca^{2+} free) FCS according to Hennings et al. (1980). Ca^{2+} concentrations were determined by atomic absorption. All media contained antibiotics (penicillin, 100 U/ml; streptomycin, 50 μg/ml). Cells were routinely cultured at 37 or 38.5°C in gassed (95% air and 5% CO_2) humidified incubators. Subcultures were obtained by disaggregating the cells with 0.1% trypsin/EDTA solution (final concentration) in Ca^{2+}/Mg^{2+}-free PBS and replating at high cell density (1:2). Later passage cells were split at a ratio of 1:5 and eventually 1:10–1:20.

DNA Fingerprinting

DNA was isolated from tissue, cultured cells, or peripheral blood by standard techniques. Digestion with Hinf I (Bethesda Research Laboratories, Gaithersburg, MD), agarose gel electrophoresis, Southern blotting, and hybridization with the hypervariable minisatellite probes 33.15 or 33.6 were performed as described by Jeffreys et al. (1985).

Growth Behavior In Vitro

Colony-forming Efficiency on Plastic. 10^3 cells were seeded per 60 mm dish (five dishes per experiment). After 2–4 wk incubation at 37°C or 38.5°C, respectively, the cultures were fixed in formalin (3.7% in PBS), stained with hematoxylin and Rhodamine, and the colonies counted macroscopically. Cloning efficiency was expressed as percentage of counted colonies to the total number of plated cells.

Population Doubling Time. Growth curves were plotted over a 7-d period. 1 × 10^5 of normal keratinocytes or 5 × 10^4 HaCaT cells were plated per 35-mm dish and three dishes were counted at daily intervals.

Cytogenetic Analysis

Semiconfluent cultures were treated for 2 h at 37°C with medium containing 0.04 to 0.08 μg/ml colcemid. The cells were detached by subsequent treatment with 0.1% EDTA (5–8 min) and EDTA/trypsin (1/1, 0.2% each; 5–8 min), centrifuged, and the cell pellet resuspended in a hypotonic solution of 75 mM KCl. After incubation for 10–15 min at room temperature, cells were fixed by three changes of methanol/acetic acid (3:1), spread on glass slides, and G-banding carried out after 2–3 wk as described (Boukamp et al., 1982). Usually 100 metaphases were analyzed microscopically and at least 20 karyograms constructed.

Growth and Differentiation Behavior In Vivo

Tumorigenicity Test. Tumor formation was assayed after subcutaneous injection of up to 5 × 10^6 cells in 100 μl culture medium into the interscapular region of 4–6-wk-old nude mice (BALB/c nu/nu backcrosses) over an observation period of at least 6 mo.

Transplantation of Cell Suspensions onto the Muscle Fascia. 1 × 10^6 normal keratinocytes or 5 × 10^5 HaCaT cells in 200 μl complete culture medium were transplanted onto the muscle fascia at the interscapular region of 6-wk-old nude mice as described (Boukamp et al., 1985). Briefly, a hatlike silicon transplantation chamber (Renner GmbH, Darmstadt, FRG) was inserted by an incision of the back skin and kept in place by tightly fixing the wound margins of the skin with wound clips. The cell suspension was seeded to the graft site by direct injection through the chamber. For histology or cryostat sections transplants were excised at weekly intervals and either fixed in buffered formalin or embedded in Tissue Tec (Lab Tec Prods, Naperville, IL) and snap frozen in liquid nitrogen, respectively.

Transplantation on Collagen Gels

Alternatively intact cultures grown on collagen substratum were transplanted essentially as described for mouse keratinocytes by Worst et al. (1982). Collagen type I from mouse tail tendon (lyophilized acetic acid extracts adjusted to 3 mg/ml) was gelled (300 μl) in the silicone culture chambers (Renner GmbH) by exposure to ammonia vapor (1.9% ammonia, 1 h) as described previously (Fusenig et al., 1982; Bohnert et al., 1986) and fixed with glutaraldehyde (4% for 1 h). After extensive washes in PBS and complete culture medium, 2 × 10^5 cells in 200 μl medium were seeded. The chambers were placed in Stanzen petri dishes (Greiner uS, Nürtingen, FRG) providing free medium access from below, and incubated at 37°C in a humidified gassed atmosphere. After 24 h cultures were rinsed and the medium covering the cells was drained. The chamber was covered with the hatlike transplantation chamber and the complete unit was transplanted onto the muscle fascia as described above.

Indirect Immunofluorescence

Cryostat sections of 5–7 μm were air-dried and incubated with the antisera listed below for 45 min at room temperature, as described by Boukamp et al. (1985). The antiserum against human involucrin was raised in rabbits (Watt, 1984) and kindly provided by Dr. Fiona Watt (Kennedy Institute of Rheumatology [JCRF], London, United Kingdom). The rabbit antiserum against rat filaggrin (Scott and Harding, 1986), which cross-reacted with the human protein, was a gift from Dr. Ian Scott (JCRF, Unilever Research, Colworth Laboratory, Bedford, U.K.). The monoclonal antibody identifying basal cells (Pab 421; Leigh et al., 1985) was kindly provided by Dr. Birgit Lane (London, UK). A rabbit antibody directed against the COOH-terminal sequence of the acidic 58K mouse keratin (corresponding to human No. 10/11; Roop et al., 1984) cross-reacting with the human 57K suprabasal keratin was donated by Dr. Dennis Roop (National Cancer Institute, Bethesda, MD). After several washes in PBS sections were labeled with the respective second antibodies, all purchased from Miles-Yeda (Rehovot, Israel), for an additional 45 min, washed in PBS and Tris-HCl buffer (pH 8.5), embedded in Aqua mount (Lerner Laboratories, New Haven, CT), and viewed under a Zeiss inverted microscope (IM 35) equipped with epifluorescence optics.

Protein Analysis

Transplants of HaCaT cells and normal human adult keratinocytes (both on glutaraldehyde-fixed collagen gels) were dissected and the epidermal tissue mechanically removed after incubation in 10 mM EDTA at 4°C for 3 h. Cytoskeletal proteins were prepared by sequential extraction using low and high salt buffers containing nonionic detergent (Triton X-100) at 4°C following our previous protocol (Breitkreutz et al., 1984, 1986). Briefly, all samples were homogenized at least twice in high salt buffer and cytoskeletons were thoroughly washed in low salt buffer and extracted in 1% SDS sample buffer (25 mM dithioerytritol, 0.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4). The samples were first homogenized on ice (glass-glass homogenizer, potter type), heated for 20 min at 90°C, homogenized once more, and after a 2-h extraction, cleared by centrifugation (40,000 *g* for 30 min at 22°C).

Two-dimensional Gel Electrophoresis. Samples dissolved in 1% SDS sample buffer were adjusted to 9.5 M urea and diluted with 2 vol sample dilution buffer (containing 9.5 M urea, 4% NP-40, 5% mercaptoethanol and 3% ampholines (pH 5–8; LKB Produkter AB, Bromma, Sweden) as described previously (Bowden et al., 1984). Separation in the first dimension was achieved by nonequilibrium pH gradient electrophoresis (NEpHGE) in tube gels using a wide ampholine range (pH 2–11) for 3,000 volthours (Vh). The gels were equilibrated in buffer containing 50 mM Tris-HCl, pH 6.8, 1% SDS, 1 mM EDTA, and 0.5% mercaptoethanol and separated in the second dimension by SDS-PAGE (7.5–17.5% polyacrylamide gradients, see Bowden et al., 1984).

Results

Development of the HaCaT Cell Line

Keratinocytes from full thickness adult human skin were generally isolated by separation of epidermis and dermis with trypsin at 4°C (Kitano and Endo, 1977) and plated at high cell density without a 3T3 feeder layer. In the case of the HaCaT cells, a histologically normal male skin specimen was obtained from the distant periphery of a melanoma (second excision). The cells were plated in high Ca^{2+} 4× MEM in 12 35-mm dishes with 1.5×10^6 cells in each. Since long-term growth in primary culture (without passaging) was optimal in low Ca^{2+} medium (0.2 mM), probably due to a marked reduction in terminal differentiation, six cultures were shifted to low Ca^{2+} medium 5 d after plating. Moreover, elevation of culture temperature (e.g., 38.5°C) had previously been shown to increase proliferative activity and to prolong the average life span from 2 up to 3 mo. Thus, all dishes were further propagated at 38.5°C.

In one culture grown in low Ca^{2+} 4× MEM at 38.5°C, a colony continued to proliferate beyond 3 mo and by 5 mo after plating had nearly covered the dish. Subculturing with routine methods (EDTA/trypsin) was unsuccessful, but cells, detached by vigorous pipetting, reattached after transfer to a new dish and grew to confluency within 4–6 wk. From this first subculture cells could subsequently be passaged by trypsinization but proliferation strictly depended on plating at high cell density (split ratio 1:2). By the fourth passage, cell density was no longer critical to maintenance of satisfactory proliferation. The resulting cell line was designated HaCaT to denote its origin from human adult skin keratinocytes propagated under low Ca^{2+} conditions and elevated temperature.

Morphologic differentiation of skin keratinocytes in culture, visible as stratification and squame formation, was usually most prominent in high Ca^{2+} medium with focal keratinization in subconfluent cultures. In low Ca^{2+} medium, cultures predominantly remained as monolayers with single cells (mostly cornified envelopes) shed into the medium as described earlier by Hawley-Nelson et al. (1980). Additionally, cell morphology changed from densely packed polygonal cells in high Ca^{2+} medium (Fig. 1 *A*) to a more heterogeneous pattern with elongated loosely arranged cells in low Ca^{2+} medium (Fig. 1 *B*).

The HaCaT cells when maintained under the initial conditions (low Ca^{2+}, 38.5°C), displayed an intermediate phenotype of polygonal cells interspersed with giant often multinucleated cells (Fig. 1 *C*) and single cornified envelopes. When the calcium level was raised (to 1.4 mM) the cultures readily stratified and formed coherent horn sheets. In contrast to their untransformed counterparts, however, the cells continued to proliferate (Fig. 1 *D*) although at a lower rate. With subsequent passages stratification and horn sheet formation steadily decreased but could be reinduced in transplants in vivo (see below). The epithelial nature of the cells under both culture conditions was confirmed by immunofluorescence with antibodies against cytokeratin as well as by their keratin profiles on one- and two-dimensional polyacrylamide gels (data not shown). Vimentin expression could not be detected by either method.

Altered Regulation of Proliferation

After prolonged cultivation HaCaT cells gradually escaped from environmentally modulated growth control in vitro and eventually became autonomous, i.e., capable of clonal growth under various culture conditions. During propagation for more than 140 passages (undergoing at least 600 population doublings) no obvious crisis was noticed.

The HaCaT cells did not show contamination with mycoplasms when tested at regular intervals with cytochemical technique (Dapi) for demonstration of DNA in infected cells. Similarly, no expression of SV40 large T antigen could be detected by immunofluorescence and Southern blot analysis under low stringent conditions excluded the presence of human papilloma virus (HPV) sequences in the HaCaT cells (M. Dürst, personal communication).

When first tested (at passage 5) HaCaT cells already exhibited altered growth properties compared with normal keratinocytes and were able to grow at clonal densities (10^3 cells per 60 mm dish, see Table I). However, at that early

1. *Abbreviation used in this paper*: NEpHGE, nonequilibrium gradient electrophoresis.

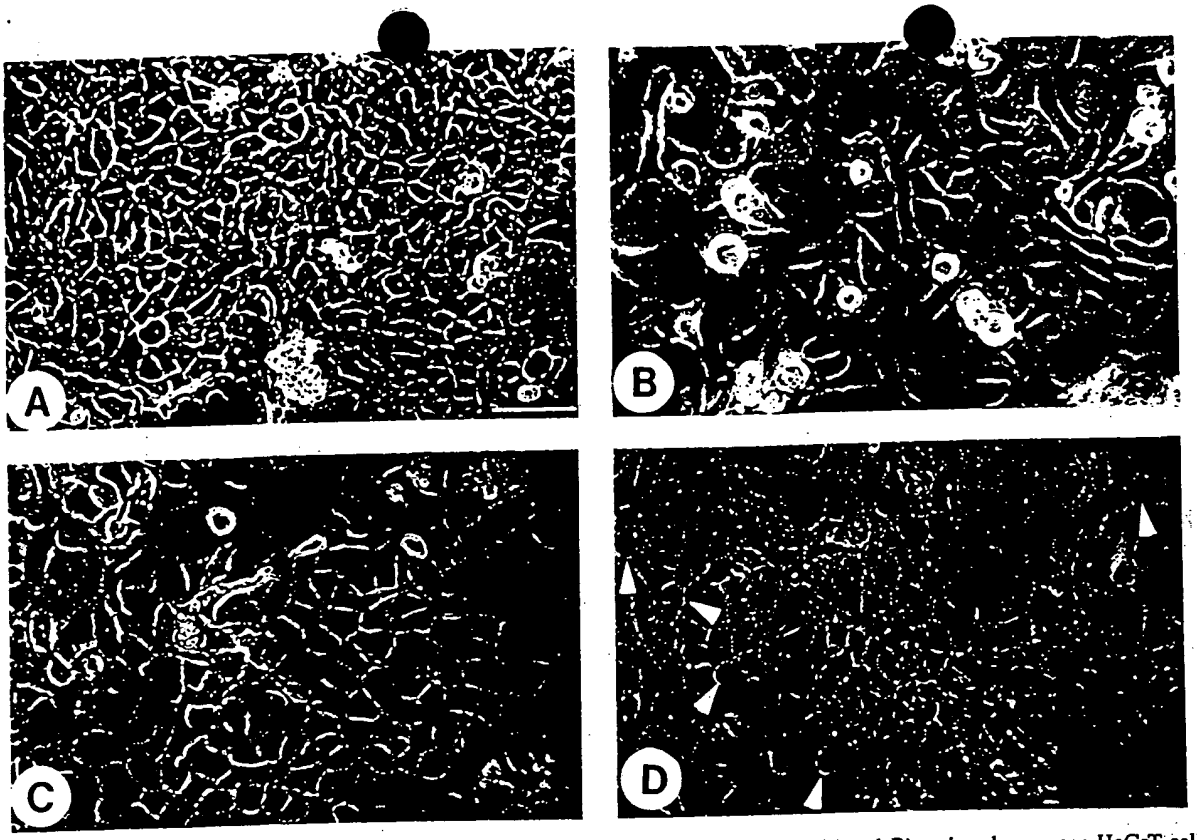


Figure 1. Phase-contrast micrographs of normal human skin keratinocytes in culture (*A* and *B*) and early passage HaCaT cells (*C* and *D*). (*A*) Normal keratinocytes grown in high Ca^{2+} 4× MEM (1.4 mM) and (*B*) in low Ca^{2+} 4× MEM (0.2 mM). (*C*) Early passage HaCaT cells grown in low Ca^{2+} 4× MEM exhibit an altered phenotype due to a more densely packed monolayer interspersed with giant, often multinucleated, cells. (*D*) After the shift to high Ca^{2+} 4× MEM HaCaT cultures are covered with coherent horn sheets but mitoses (*arrowheads*) remain frequent. (Bar, 50 μm).

stage the cells were still very sensitive to modifications of the original culture conditions (0.2 mM Ca^{2+}, 38.5°C). Reduction of the culture temperature (to 37°C) or elevation of the Ca^{2+} level (to 1.4 mM Ca^{2+}) resulted in a drastic decrease in cloning efficiency and a prolonged population doubling time. Around the tenth passage cells developed complete independence of both Ca^{2+} concentration and temperature. With further passages, however, cloning efficiency and growth rate did not change significantly.

The ability to grow in soft agar appeared to be a relatively late event and was first noticed at passage 18 although with a low efficiency that subsequently remained unchanged. Agar colonies were viable as judged by staining and replating of selected clones.

Although the HaCaT cell line exhibited a transformed phenotype in vitro, was clonogenic on solid and in semisolid substrata, and presumably immortalized, cells were neither tumorigenic after subcutaneous injection (up to passage 100, so far tested) nor invasive in a more sensitive transplantation assay (see Boukamp et al., 1985). Small nodules that developed shortly after injection regressed within 3–6 wk and late recurrences were not seen within 12 mo of observation.

Cytogenetic Characteristics

The chromosomal constitution of HaCaT cells was followed during propagation, starting with passage 2, when the cells had been in culture for a total of 8 mo. At this early stage distinct numerical and structural karyotypic alterations were obvious (Table II). The majority of cells were hypodiploid with an average of 44 chromosomes resulting from full or partial monosomies of chromosomes involved in the formation of marker chromosomes. All metaphases had the XO sex chromosome constitution (lacking the Y chromosome) and were partially monosomic for the short arm of chromosome Nos. 3, 9, and 4 (later monosomy of whole No. 4) while trisomic for the long arm of No. 9 (due to i[9q]). Three individual marker chromosomes (M1, t(3q4q); M2, i(9q); and M3, del(4q28)) were present in 100% of the metaphases and clearly indicated the clonal origin of this line (Fig. 2 *A*). Moreover, these individual cytogenetic characteristics excluded cross-contamination with other human cell lines developed and/or used in our laboratory.

Starting at passage 5 a hypotetraploid stem line evolved with a range of 72–88 chromosomes (including duplication of the early markers) and a fourth marker chromosome (M4 [4p18q]) appeared, replacing M3 (Fig. 2 *B*). These cytogenetic alterations were apparently linked to changes in growth control, indicated by improved adaptation to growth under modified culture conditions. With further passages additional markers developed, mainly involving chromosome Nos. 1, 6, 15, 17, and 22. However, these late structural aberrations were found in only 15–50% of metaphases.

Identity of HaCaT Cell and Donor DNA

To prove the origin of the HaCaT cell line from the original donor and to exclude cross-contamination with other human

Table I. Adaptation of the HaCaT Cell Line to Autonomous Growth during In Vitro Propagation

Culture passages	Culture temperature	Cloning efficiency on plastic	Population doubling time	Cloning efficiency in soft agar	Tumorigenicity
n	°C	%	*h*	%*	
5	38.5	7.5	26 (0.2 mM Ca^{++})		
	37	0.9	50	ND	–
7	38.5	ND	38	ND	–
	37		39		
11	37	7.8	ND	ND	ND
15	37	10.8	22	–	ND
18	37	ND	ND	0.24	–
29	37	14.2	ND	0.27	–
37	37	13.3	23	0.27	–
48	37	13.2	23	0.36	–
81	37	ND	21	ND	–

* As tested in high Ca^{++} 4× MEM (1.4 mM) unless specified.
ND, not done.

cell lines (in addition to evidence from karyotyping), HaCaT cells at various passage levels were analyzed by DNA fingerprinting (Jeffreys et al., 1985). Using the hypervariable minisatellite probes 33.15 and 33.6, we demonstrated the identity of the DNA fingerprints obtained from the donor tissue with that of the HaCaT cells. Moreover, despite the documented drastic cytogenetic changes during prolonged cultivation of the HaCaT cell line, the highly characteristic DNA fingerprint pattern remained unchanged from passages 6–79 (Fig. 3). The results were absolutely reproducible and identical patterns were obtained in five independent analyses. For further confirmation, DNA fingerprints prepared from eight other cell lines (previously developed or grown in our laboratory, including HeLa) and two different patients' blood samples were all unique and showed no similarity to the pattern of the HaCaT cells (see Fig. 3).

Growth and Differentiation Behavior In Vivo

To date, complete differentiation of keratinocytes, including regular tissue organization and normal expression of differentiation products, has only been shown to occur under mesenchymal influence (Worst et al., 1982; Lavker and Sun, 1983; Breitkreutz et al., 1984, 1986; Bohnert et al., 1986; Fusenig, 1986). Thus, HaCaT cells were further analyzed for their differentiation potential after injection into or transplantation onto athymic nude mice. When injected subcutaneously cells developed nodules that, with later passages, occasionally persisted over several weeks. Histological sections of these nodules revealed large encapsulated cysts often filled with horny squames, while the lining epithelium was rather thin or atrophic (data not shown).

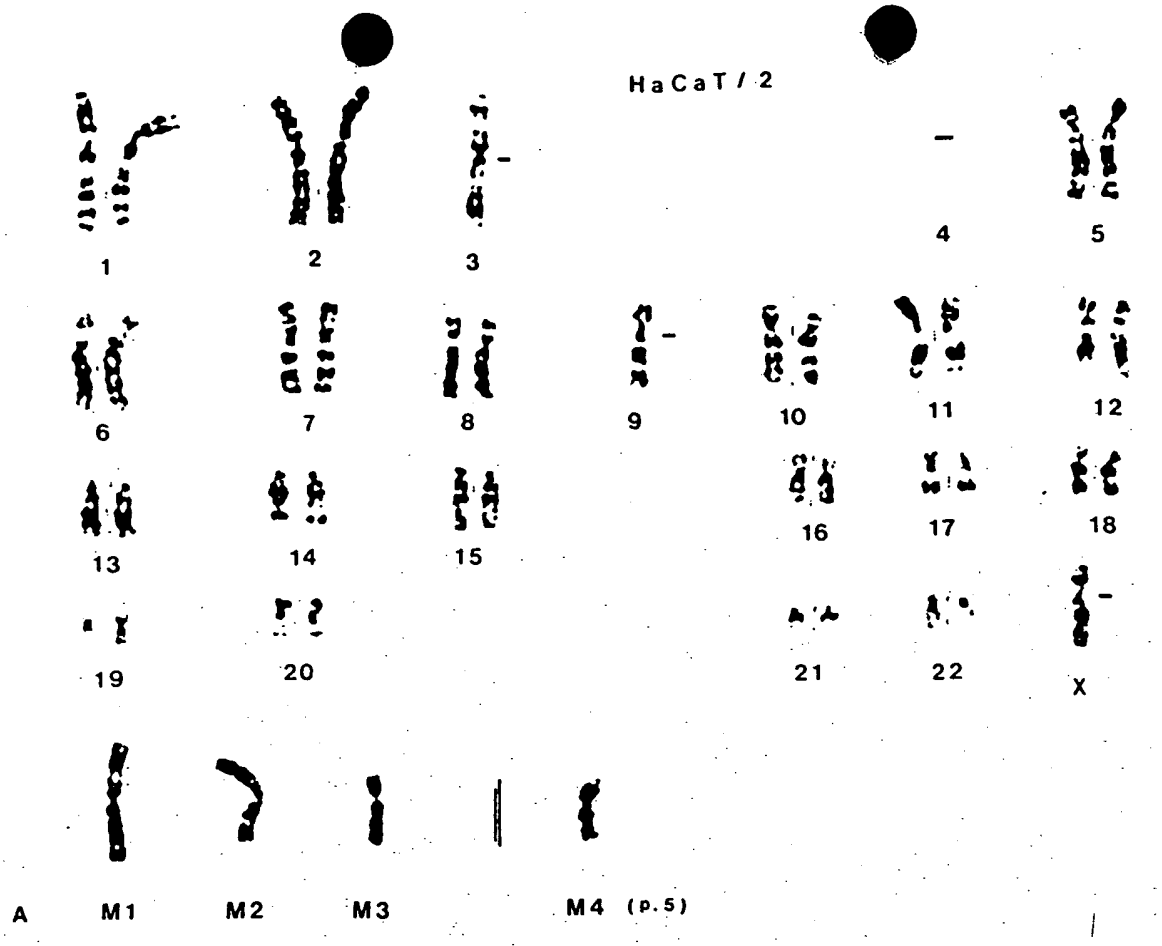
Similar encapsulated cystic structures were formed when suspensions of HaCaT cells were transplanted onto the subdermal muscle fascia as described for human skin carcinoma cells (Boukamp et al., 1985). 1–2 wk after transplantation the lining epithelium of these cysts had developed a regular tissue architecture including stratum granulosum and corneum (Fig. 4 *A*). The living epithelium eventually degenerated, leaving behind a cyst filled with horny material. This growth and differentiation pattern, characteristic for all passage levels tested (up to 80), was very similar to that of transplanted normal human skin keratinocytes.

When transplanted as intact cultures growing on glutaraldehyde-fixed collagen gels, contact between keratinocytes and host tissue (supposedly leading to encapsulation of the epithelial cells) was prevented. Under these circumstances HaCaT cells reconstituted an almost perfect epidermis (Fig. 4 *B*) similar to that formed by normal adult keratinocytes (Fig. 4 *C*). However, normal tissue architecture was only observed after a delay of 1–2 wk in HaCaT transplants, indicating reduced sensitivity to environmental signals. With later passages the epithelium formed in vivo showed slight devia-

Table II. Chromosomal Changes of HaCaT Cells during Adaptation to Autonomous Growth In Vitro

Passage no.	Numerical distribution (percent of metaphases)			Marker chromosomes* (percent of metaphases)				
	Diploid (46)	Hypodiploid (38–45)	Hypotetraploid (72–88)	M1	M2	M3‡	M4‡	M5-M8§
2	10	90	0	100	100	100	0	0
5	0	67	33	100	100	77	23	5
11	0	58	42	100	100	70	30	8
17	0	0	100	100	100	0	100	25
33	0	0	100	100	100	0	100	100‖
50	0	0	100	100	100	0	96	100¶

* M1 t(3,4)(qter → q11:q11 → qter), M2 i(9)(qter → q11:q11 → qter), M3 del(4)(q28 → qter), M4 (4,18)(pter → p11:q11 → qter).
‡ Metaphases contained only either M3 or M4 alternatively.
§ M5: dup(1)(q 23.1 → 25.3); M6: t(15,22); M7: dup(6)(p22); M8: dup(17)(q23.1 → q25.3).
‖ Present in 47, 30, 30, and 15% and
¶ Present in 50, 30, 25, and 25% of the metaphases, respectively.


HaCaT / 2
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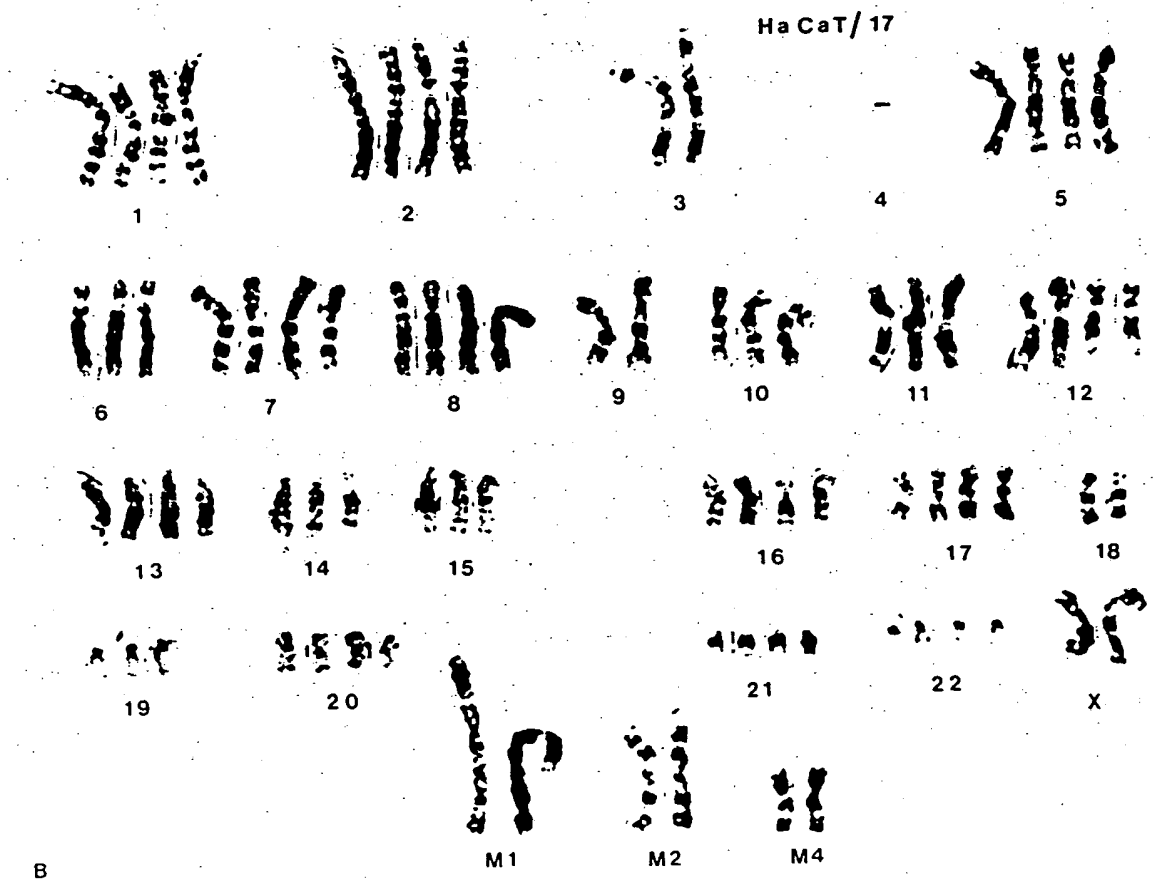
HaCaT/17
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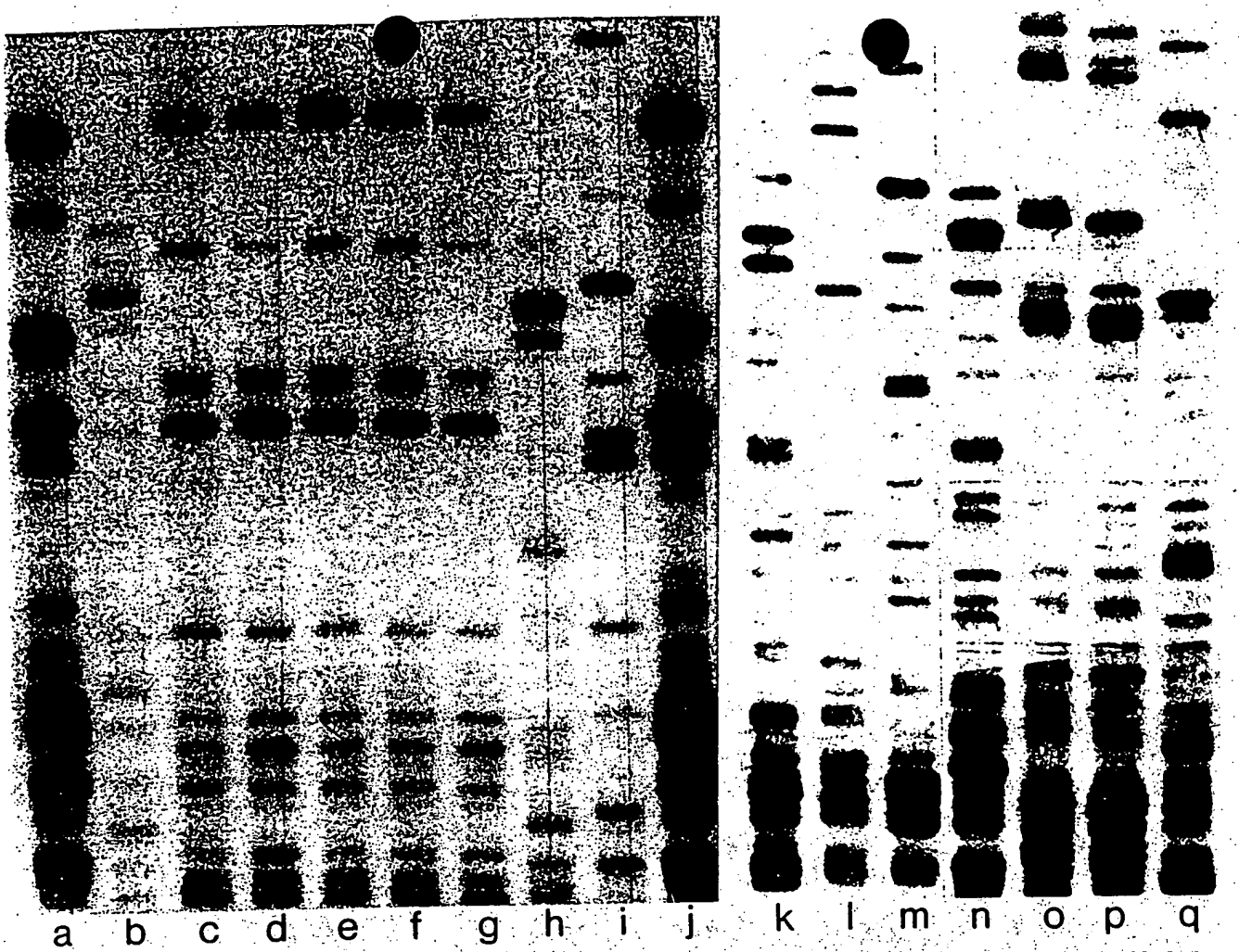

Figure 3. Southern blot analysis of DNA from tissue and cell samples hybridized with the hypervariable minisatellite probe 33.15. (lane *a*) and standard DNA from an unrelated subject (lane *j*); (lane *b*) lymphocyte DNA from an unrelated patient; (lane *c*) skin biopsy of donor patient (EB); (lanes *d–g*) HaCaT cells from passages 45, 6, 16, and 79, respectively; (lane *h*) HaSV cells, human keratinocyte line immortalized by transfection with SV40 DNA, passage 18; (lane *i*) HeLa cells; (lane *k*) HaSV cells, passage 43; (lane *l*) SCL-I cells and (lane *m*) SCL-II cells, established from squamous cell carcinomas of the skin; (lane *n*) TR 126 cells; (lane *o*) TR 131 cells; (lane *p*) TR 138 cells; (lane *q*) TR 146 cells (lanes *n–q* established from squamous cell carcinomas of the head and neck; see Boukamp et al., 1985).

tions from regular tissue architecture and an increasing transition from an ortho- to a parakeratotic stratum corneum (containing nuclear remnants). However, a distinct stratum granulosum (usually restricted to orthokeratinization) was present in all transplants.

Using other differentiation markers in an effort to further substantiate the histological findings, transplants of HaCaT cells at different passage levels were analyzed both biochemically and by immunofluorescence. Involucrin, a major precursor protein of the cross-linked envelopes in the stratum corneum, was clearly restricted to the upper flattened layers of the transplants (Fig. 4 *D*), a characteristic feature of long-term transplants of normal adult keratinocytes (Watt et al., 1987). Also filaggrin, the major component of keratohyalin granules, was detected in the two uppermost living layers (Fig. 4 *E*), i.e., the stratum granulosum. Moreover, basal and suprabasal cell compartments were clearly distinguishable (as in the epidermis) by a monoclonal antibody (Pab421) specific for epidermal basal cells (Fig. 4 *F*) and by a polyclonal antibody specific for one of the epidermal suprabasal keratins (57 kD, No. 10/11 according to Moll et al., 1982; Fig. 4 *G*).

These findings correlated well with keratin analysis by one- and two-dimensional gel electrophoresis (NEpHGE/SDS-PAGE). When transplants of HaCaT cells (Fig. 5 *A*) were compared with those of normal cells derived from adult foreskin (Fig. 5 *B*) very similar patterns were observed. As in the human epidermis (adult thigh skin, Fig. 5 *C*) the

Figure 2. G-banded karyotype of the HaCaT cells. (*A*) At passage 2 the cells revealed a hypodiploid stem line (mode of 44 chromosomes) with three individual marker chromosomes: M1, t(3q4q); M2, i(9q); M3, del(4q28) (in 100% of the metaphases) and monosomy X. At passage 5 the marker M4 t(4p18q) appeared. (*B*) At passage 17 the stem line shifted to hypotetraploidy (mode of 82). The markers M1, M2, and M4 were present in two copies while M3 had disappeared.

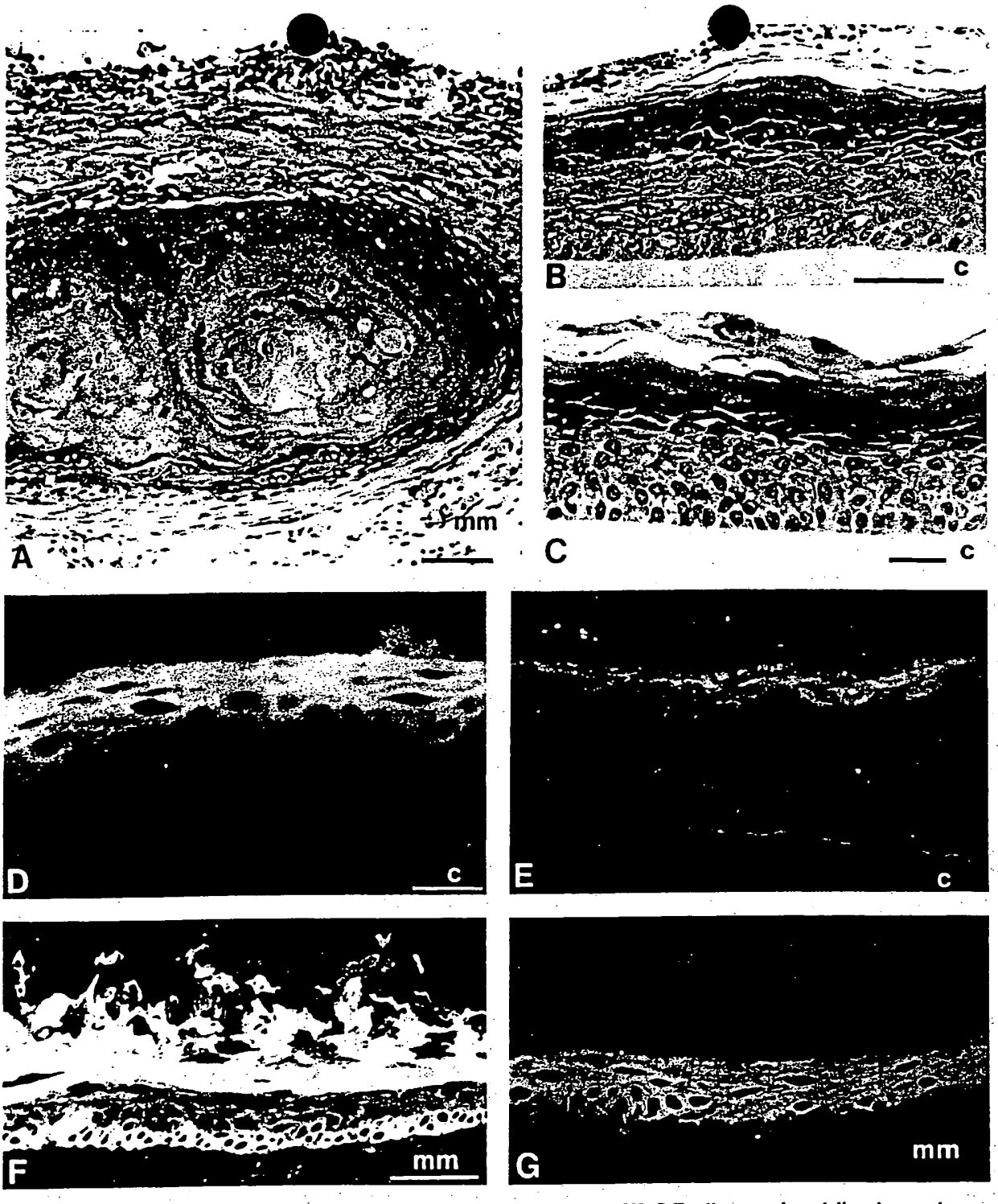

Figure 4. Histological sections of 2-wk-old HaCaT transplants. (*A*) Suspensions of HaCaT cells, transplanted directly onto the muscle fascia of nude mice, develop cysts within the mouse mesenchyme (*mm*) with orderly stratified keratinizing epithelium. (*B*) HaCaT cells transplanted as intact cultures on collagen substratum (*c*) form a regular epidermis comparable (*C*) to normal skin keratinocytes. Immunofluorescence of HaCaT transplants on collagen (*D* and *E*) and directly on the muscle fascia (*F* and *G*). (*D*) Antiserum against human involucrin shows a membrane-bound reaction in the upper flattened living cell layers of the epithelium. (*E*) Filaggrin staining (spotlike fluorescence) apparent in the uppermost living cell layers corresponding to the keratohyalin granules of the stratum granulosum. (*F*) The lowermost cell layer of the epithelium lining the cysts in the mouse mesenchyme (*mm*) is stained with a basal cell-specific monoclonal antibody (Pab421). The stratum corneum is unspecifically labeled by FITC. (*G*) The suprabasal cell layers of the epithelium are stained with sequence-specific polyclonal antibodies against the high molecular mass acidic 57-kD (No. 10) keratin. Bars: (*A*) 100 μm; (*B*, *F*, and *G*) 50 μm; (*C*, *D*, and *E*) 25 μm.

suprabasal basic 68-kD and acidic [redacted]kD keratins (Nos. 1 and 10/11), as well as the basal basic [redacted]kD and acidic 51-kD keratins (Nos. 5 and 14, respectively) were strongly expressed. Furthermore, both HaCaT and normal cell transplants maintained the hyperproliferative set of basic 58-kD and acidic 49-kD keratins (Nos. 6 and 16, respectively). Minor components were keratin No. 4 (presumably) and No. 13. Keratin Nos. 6 and 16 are regularly expressed in culture (together with the basal keratin pair and No. 17), but in epidermis only in the hyperplastic state (Sun et al., 1983). These observations are compatible with the general (hyperplastic) morphology of transplants and consistent with previous data on transplanted mouse keratinocytes (Breitkreutz et al., 1984, 1986).

Keratin No. 13, abundant in foreskin and derived cultures but absent in adult epidermis, was prominent in HaCaT cultures (data not shown). Also present, although mostly in traces, were keratin Nos. 7, 8, 15, 18, and 19. These simple epithelial-type keratins were highly inducible by vitamin A in vitro but absent in transplants. Interestingly, in vitamin A–deprived cultures (delipidized serum) HaCaT cells expressed keratin patterns comparable to those of transplants (our unpublished data). This further stresses the similarity of the differentiation behavior of HaCaT cells to that of normal keratinocytes. Generally this keratinization pattern is in marked contrast to SV40-transformed keratinocyte lines where small or simple epithelial-type keratins such as 7, 8, and 18 or 19 clearly predominate (Bernard et al., 1985; Steinberg and Defendi, 1983; Banks-Schlegel and Hawley, 1983; our unpublished observations) and where the suprabasal keratin pair is apparently not induced in vivo.

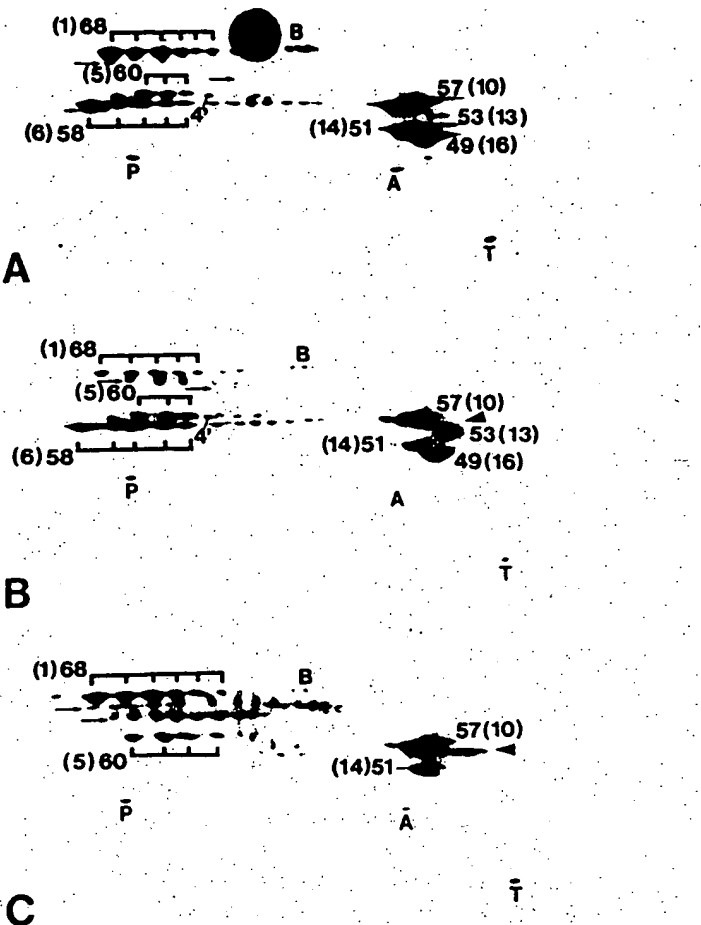

Figure 5. Keratins expressed in cell transplants (*A* and *B*) and human epidermis (*C*) separated on two-dimensional gels. In both transplants (*A*) of HaCaT cells and (*B*) those of normal human keratinocytes the suprabasal (68 kD, No. 1 and 57 kD, No. 10/11) and basal keratins (60 kD, No. 5 and 51 kD, No. 14) are present at about equal levels. In addition, the hyperproliferative set (58 kD, No. 6 and 49 kD, No. 16) is expressed, which is generally lacking in normal adult (thigh) epidermis (*C*). The varying degrees of turnover products (derived from keratin No. 1, marked by small arrows; also from No. 10/11, indicated by some slight trailing to the right, arrowhead) between the transplants are also observed in normal epidermis of different individuals and/or depending on body location. The minor spots in the neutral range (*A*, possibly also *B*; marked 4') might represent some keratin No. 4 (assessed by comigration with authentic sample). Separation in the first dimension was achieved by NEpHGE (*right to left*) and in the second dimension by SDS-PAGE (7.5–17.5% acrylamide gradient). Markers included are: *B*, BSA (68 kD, pI 6.4); *P*, 3-phosphoglycerate kinase (43 kD, pI 7.4); *A*, α-actin (42 kD, pI 5.4); *T*, tropomyosin (36 and 38 kD, pI 4.9).

Discussion

Herein we have reported the spontaneous transformation in vitro of human keratinocytes from histologically normal adult body skin. The apparently immortalized but highly differentiated cell line was named HaCaT to indicate the origin and initial growth conditions (see Experimental Procedures). The line is clonal in origin as indicated by specific and stable cytogenetic markers, has a transformed phenotype in vitro but is not tumorigenic, and is noninvasive in vivo. This clearly demonstrates, in accord with earlier studies (Azzarone et al., 1976; Danes and Surano, 1982; Mukherji et al., 1984; Thielmann et al., 1983; Revoltella et al., 1986; Baden et al., 1987*a*, *b*; Nagasawa et al., 1987), that spontaneous transformation of human cells in vitro can occur, although the number of reported cases is still rather low.

The relatively low success rate for transformation of human cells in vitro has mainly been attributed to the higher genetic stability of human cells as compared with rodent cells (DiPaolo, 1983; Sager et al., 1983). Although detailed studies on chromosomal alterations using banding techniques are rare, it has been shown that cytogenetically abnormal clones in cultures of adult human skin fibroblasts are by no means uncommon (Littlefield and Mailhes, 1975; Harnden et al., 1976; Nagasawa et al., 1987). Our preliminary experiments with normal human keratinocytes indicate that numerical (mainly polyploidization) and nonspecific structural chromosomal changes are present in early and late primary cultures as well as subcultures (R. T. Petrusevska, unpublished observations). Other studies with cultured rat embryo and monkey fibroblasts indicated that elevated temperature (as used here to establish the cell line) accelerates the occurrence of (spontaneous) chromosomal aberrations (Marczynska et al., 1980). We are currently investigating this effect in primary and low passage human keratinocyte cultures.

The significance of chromosomal alterations in different stages in cell transformation is not yet fully established. The early appearance and the stability of the distinct cytogenetic

changes (particularly the unbalanced hypodiploidy) in the HaCaT cells strongly indicate that they are essential for the early disturbances in growth control resulting in prolonged or unlimited life span. The subsequent occurrence of a hypotetraploid HaCaT subclone and its correlation with improved growth capacity in vitro has also been previously observed in two human skin carcinoma cell lines (Tilgen et al., 1983) and in transformed mouse keratinocyte lines (Fusenig et al., 1985). Therefore, polyploidization, which further contributes to genomic imbalance and altered gene expression, may be crucial for the acquisition of autonomous growth in culture but is certainly not sufficient for malignant conversion. Very recently, another spontaneous human keratinocyte cell line (from foreskin) that exhibited as a sole karyotypical abnormality a trisomy of chromosome No. 8 has been described (Baden et al., 1987*a*, *b*). The fact that this near diploid cell line still depends on 3T3 feeder support for continuous growth, further supports the hypothesis that polyploidization is crucial for autonomous growth in vitro.

Because spontaneous immortalization is still generally considered to not occur in human cells, it was critical to prove the identity of the HaCaT cells with the original donor and to exclude cross-contamination with epithelial human cell lines used in our laboratory. Although the cytogenetic data documenting individual marker chromosomes had virtually excluded possible cross-contamination, additional proof was highly desirable. DNA fingerprinting on Southern blots using hypervariable minisatellite probes (Jeffreys et al., 1985) seemed to be the method of choice, although it had not previously been demonstrated whether chromosomal alterations would influence the applicability of this technique. The results unequivocally proved that despite the transformed phenotype and multiple structural chromosomal changes the DNA fingerprint pattern remained essentially identical at various passage levels and was clearly identical to a DNA sample from the patient. Moreover, the nonidentity of a variety of different cell lines tested as well as DNA fingerprints from 200 unrelated individuals (data not shown) clearly demonstrated the unique DNA fingerprint pattern of HaCaT cells and their original donor.

These findings underline the power of the DNA fingerprinting technique for unequivocal identification of cultured cells and the verification of their original donor, irrespective of the tissue used as DNA source (e.g., lymphocytes, skin tissue sample, and keratinocytes). Thus, these results provide new standards for the identification of human cells in vitro irrespective of time in culture and genetic alterations.

It is a widely accepted postulate that alterations in differentiation are essential for the transformation process and that malignancy and differentiation are inversely correlated (Broders, 1932). Cell lines derived from skin carcinomas usually exhibit defects in their differentiation capacity, although with considerable variations (Rheinwald and Beckett, 1980; Boukamp et al., 1985). In accord with this, immortalization of human keratinocytes with SV40 was accompanied by drastic alterations in differentiation potential (Banks-Schlegel and Howley, 1983; Steinberg and Defendi, 1983; Bernard et al., 1985). It has therefore been stated that "inhibition of keratinization might be an early step in carcinogenesis." Steinberg and Defendi (1983) and concluded that the loss in differentiation capacity in cell lines would be "the price one has to pay for immortalization." However, these properties of the SV40-transformed cells might be virus-related rather than linked to transformation per se. This is in line with our own data on SV40-immortalized human skin keratinocytes (our unpublished results) and other studies on virus-transformed keratinocytes (Yuspa et al., 1983; Weissman and Aaronson, 1985).

In contrast to these virally transformed human cells, the HaCaT cells, although immortal, have largely retained their capacity to reconstitute a well structured epidermis after transplantation in vivo. The virtually normal degree of morphologic differentiation was further substantiated by the regular spatial distribution of epidermal differentiation products. In addition, the pattern of keratin expression, including the suprabasal epidermal keratins (Nos. 1 and 10), was almost identical to those seen in transplants of normal keratinocytes. We have previously reported similar findings on a series of spontaneously transformed mouse keratinocyte lines (Breitkreutz et al., 1986). The HaCaT cells also maintained these properties at higher passage levels, even though differentiation in vitro (stratification and squame formation) gradually decreased and slight alterations in tissue architecture occurred in vivo. In addition, HaCaT cells from passage 80 or higher showed a comparable induction of suprabasal keratins in response to vitamin A depletion in the culture medium (our unpublished results). In this way, the HaCaT cell line is closely approximated to normal keratinocytes, and thus offers a suitable model to study regulatory mechanisms in the process of differentiation of human epidermal cells. Moreover, since HaCaT cells could be reproducibly and efficiently transfected with the human Ha-ras-1 oncogene (EJ), giving rise to clones with abnormal growth properties in vivo, including tumorigenesis (Fusenig et al., 1987; Boukamp, P., D. Breitkreutz, E. Stanbridge, P. Cerutti, and N. Fusening, manuscript in preparation), this cell line provides a valuable model system for the study of the role of oncogenes and other factors in the process of malignant conversion of human epithelial cells.

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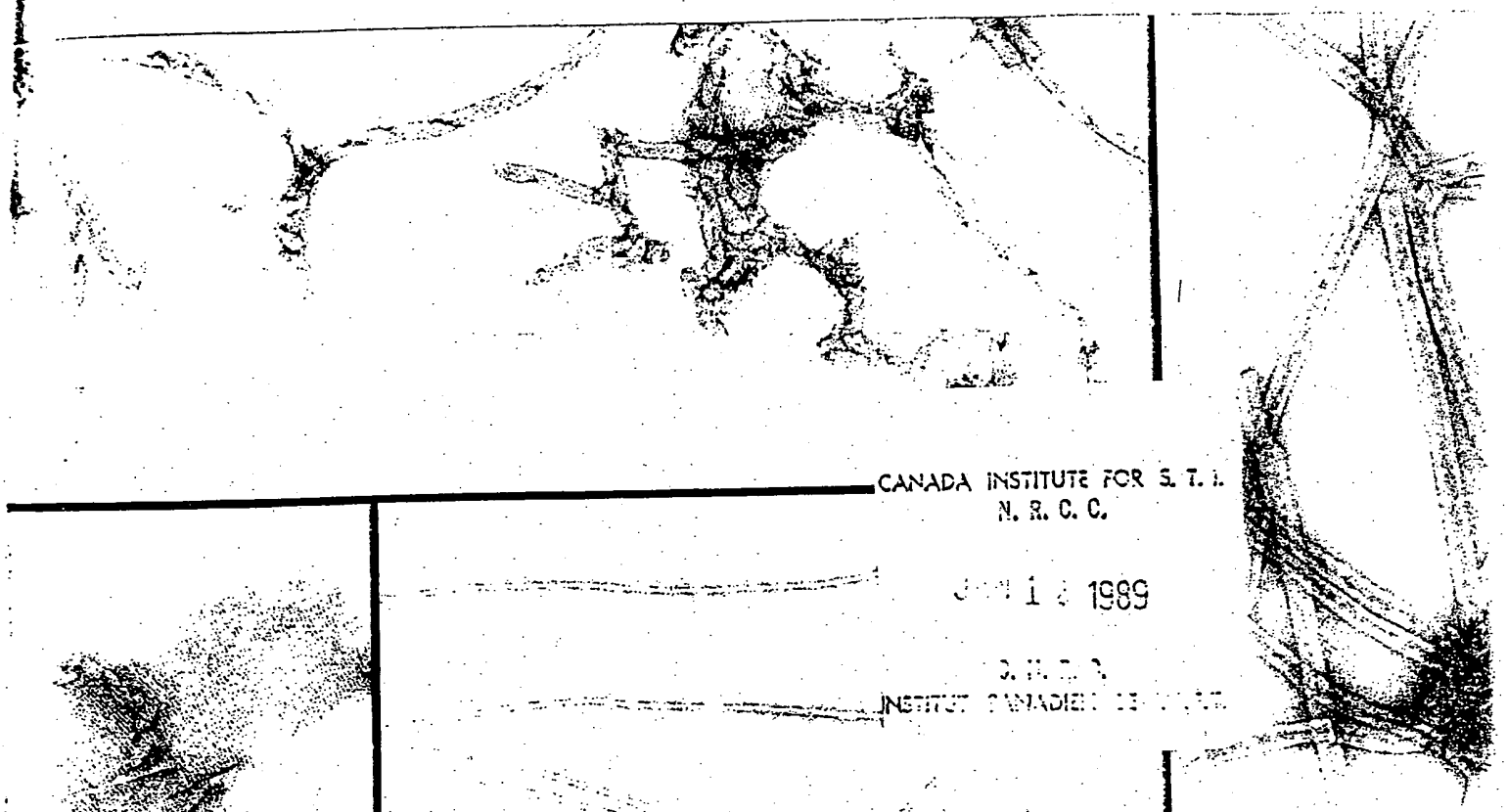

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In Vitro Infection and Type-Restricted Antibody-Mediated Neutralization of Authentic Human Papillomavirus Type 16

WENDY I. WHITE,[1]* SUSAN D. WILSON,[1] WILLIAM BONNEZ,[2] ROBERT C. ROSE,[2] SCOTT KOENIG,[1] AND JoANN A. SUZICH[1]

MedImmune, Inc., Gaithersburg, Maryland 20878,[1] and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642[2]

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Human papillomavirus type 16 (HPV-16) is strongly associated with the development of cervical cancer. Studies of model systems with animal papillomaviruses have demonstrated the importance of neutralizing antibodies in preventing papillomavirus-associated disease. The assessment of neutralizing antibody responses against HPV-16, previously hampered by the lack of a viral source, was enabled by the recent propagation of an HPV-16 stock in xenografted severe combined immunodeficiency (SCID) mice. HPV-16 infection of an immortalized human keratinocyte cell line was demonstrated by detection of an HPV-16-specific spliced mRNA amplified by reverse transcriptase PCR. Infection was blocked by preincubation of the virus with antiserum generated against HPV-16 virus-like particles (VLPs) composed of the major capsid protein, L1. To examine potential cross-neutralizing activity among the different genital HPV types, rabbit antisera to L1 VLPs corresponding to HPV-6, -11, -18, -31, -33, -35, -39, and -45 were assayed for the ability to block the HPV-16 infection of cultured cells. Antiserum raised against HPV-33 L1 VLPs was the only heterologous antiserum which inhibited HPV-16 infection. Thus, a neutralization assay for HPV-16 may help to characterize the components required to compose a broadly efficacious genital HPV vaccine.

Human papillomaviruses (HPVs) are the most common sexually transmitted viral pathogens in the United States (26). "Low-risk" HPVs such as HPV-6 and -11 are associated with the production of benign genital warts, while "high-risk" types such as HPV-16 and -18 are known to be a major causative factor in the development of cervical cancer. The association of cervical carcinogenesis and HPV infection is indicated by strong epidemiological evidence and the detection of HPV DNA in more than 93% of cervical cancers from all geographic areas (5). Of the high-risk types, HPV-16 is the most prevalent, being present in 50% of cervical tumor specimens worldwide. Other high-risk HPV types include HPV-18, -31, -33, and -45.

Due to the morbidity and mortality associated with the high-risk HPV types, there is keen interest in developing prophylactic HPV vaccines. Results obtained with several different animal models (canine oral papillomavirus, cottontail rabbit papillomavirus [CRPV], and bovine papillomavirus type 4 [BPV-4]) established the feasibility of developing vaccines to prevent papillomavirus disease (7, 19, 35). These animal studies demonstrated the protective efficacy of the major papillomavirus capsid component, the L1 protein. When expressed in eukaryotic cells, the L1 proteins of many different papillomavirus types self-assemble into virus-like particles (VLPs) that are antigenically and morphologically similar to authentic papillomavirions (16, 18, 31). Animals immunized with L1 VLPs were protected from subsequent papillomavirus challenge. Successful vaccination required that the VLPs be composed of the L1 protein of the challenge virus, and immunity was found to be generally type specific. In both the canine oral papillomavirus and CRPV animal models, passive transfer of immune serum from VLP-immunized animals to naive animals conferred protection from subsequent challenge with the homologous papillomavirus, suggesting that antibodies serve as a major protective component against papillomavirus infection (7, 35).

The results with animal models provide a strong rationale for the development of VLP-based vaccines to prevent HPV-induced genital warts and cervical cancer. However, HPV vaccine development has been hindered by the high degree of species specificity exhibited by these viruses, which has made direct evaluation of vaccine efficacy in animals impossible. Also, difficulties in the propagation of HPV stocks have hampered the examination of neutralizing antibody responses against authentic HPVs.

One notable exception is the low-risk HPV-11, which has been propagated with a xenograft system in a sufficient quantity to allow direct evaluation of neutralizing antibodies (12, 14, 20). Antisera generated against HPV-11 VLPs have been shown to contain high titers of HPV-11-neutralizing antibodies, as assessed by the abrogation of condyloma growth in the xenograft system. Recently, a method was developed to study antibody-mediated neutralization of HPV-11 in vitro (34). In this assay, HPV-11 infection of cultured human keratinocytes was determined by the appearance of an HPV-11-specific mRNA detected by reverse transcriptase PCR (RT-PCR). Preincubation of the virus with antibodies which had previously been shown to neutralize HPV-11 in the xenograft assay prevented HPV-11 infection of the keratinocytes, as demonstrated by the inability to detect HPV-11-specific transcripts.

The lack of a reliable source of virus has prevented the direct evaluation of neutralizing antibodies specific for the high-risk HPV-16. Researchers have relied on surrogate assays, such as inhibition of VLP-mediated hemagglutination, to study the functional activity of antisera generated against HPV-16 VLPs (28). Recently, HPV-16 has been propagated with a SCID mouse xenograft system (2). In the present study, we demonstrate that an HPV-16 stock prepared from the xenografted condylomas can infect an immortalized keratinocyte cell line in vitro, as measured by the appearance of an HPV-16-specific

* Corresponding author. Mailing address: 35 W. Watkins Mill Rd., Gaithersburg, MD 20878. Phone: (301) 417-0770. Fax: (301) 527-4200. E-mail: whitew@medimmune.com.

transcript. HPV type-specific antibodies inhibited HPV-16 infection in vitro, thus providing the first direct evidence of antibody-mediated neutralization of an authentic high-risk HPV. In addition, the potential for cross-protection among the high-risk and low-risk genital HPV types was assessed by examining the ability of antisera to VLPs of various heterologous HPV types to neutralize HPV-16 infection.

MATERIALS AND METHODS

Isolation and propagation of HPV-16. Our HPV-16 strain was isolated and propagated with the xenograft SCID mouse model (3, 20). The propagation process and the typing analyses of viral stock passages are described in detail elsewhere (2). In brief, single biopsy samples were obtained from 11 patients with clinical condylomata acuminata. Subsequent histologic diagnosis showed that two of the patients had mild to moderate intraepithelial neoplasia. The biopsy samples were ground in phosphate-buffered saline (PBS) with sand and a mortar and pestle. The preparation was submitted to low-speed centrifugation, and the supernatant was pelleted at 100,000 × *g* for 1 h at 4°C and resuspended in PBS. This viral suspension was used to infect neonatal human foreskin fragments that were each implanted under the renal capsule of three SCID mice (3, 20). Twelve weeks later, the mice were sacrificed and the grafts were collected. One of the six grafts showed histologic evidence of intraepithelial neoplasia and was prepared as described above to make a lysate for a second passage. Twelve SCID mice were grafted under the renal capsule with neonatal foreskin fragments (one per kidney) that had been incubated in the lysate. The mice were sacrificed 19 weeks later. Five of 15 retrieved grafts had evidence of HPV infection by histology, and one of them contained HPV capsid protein by immunocytochemistry. The remaining tissue samples from the grafts were used to prepare a viral lysate as described above. HPV capsids were demonstrated after negative staining by electron microscopy. The viral DNA was extracted from the lysate and subjected to PCR analysis with the MY09/MY11 primer pair (21). The PCR fragment was cloned and sequenced. The DNA sequence was identical to a reference strain of HPV-16 (32), except at seven nucleotide positions. Five of the base changes resulted in no amino acid substitution, one caused a conservative change (threonine to serine [A6801T]), and one yielded a nonconservative substitution (threonine to proline [A6693C]). The viral lysate was used for a third passage of the virus. Single infected grafts were implanted under each renal capsule as well as under the skin of each flank of 24 SCID mice. The animals were sacrificed 27 weeks later, and the renal and subcutaneous grafts were collected to prepare a viral lysate as described above. The virus stock was typed by amplification of viral DNA with the MY09/MY11 primers and hybridization with oligonucleotide probes specific for the following HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 59, 66, 68, 73, MM4, LVX-82/MM7, and MM8 (1, 17, 22). Positive results were obtained only with the HPV-16-specific probes. The MY09/MY11 amplicon was also typed by digestion with the restriction enzymes *Eco*RI and *Pst*I, which resulted in a banding pattern consistent with HPV-16 (23). This virus stock was designated HPV-16 $_{\text{Rochester-1k/ur3}}$ and was used in the present experiments.

Antisera to VLPs. Polyclonal rabbit antisera specific for HPV-11, -16, and -18 L1 VLPs have been described previously (30). Antisera to HPV-6 L1 VLPs were kindly provided by S.-J. Ghim. To generate antibodies against HPV-31, -33, -35, -39, and -45 L1 VLPs, genomic clones of HPV-31 and HPV-35 were obtained from the American Type Culture Collection (Rockville, Md.); HPV-33 and HPV-39 DNAs were provided by M. Favre and G. Orth (Institut Pasteur, Paris, France); and cloned HPV-45 DNA was provided by A. Lorincz (Digene Diagnostics, Inc., Silver Spring, Md.). PCR amplification of the L1 sequences and generation of recombinant baculoviruses carrying genes encoding L1 were carried out as previously described (30). VLPs composed of HPV-31, -33, -35, -39, and -45 L1 were purified from recombinant baculovirus-infected Sf9 or High Five cells on CsCl density gradients. Total protein concentrations were determined with a commercial assay (bicinchoninic acid; Pierce Chemical Co., Rockford, Ill.). For each HPV VLP type, a New Zealand White rabbit was immunized intramuscularly at two sites with an emulsion of 50 μg of L1 protein in complete Freund's adjuvant and given a booster injection 2 weeks later with an emulsion prepared with the same VLP type and incomplete Freund's adjuvant. The reactivity of the antisera against the homotypic VLPs used for immunization was determined by enzyme-linked immunosorbent assay (ELISA), with the titer being defined as the greatest dilution which yielded an optical density value greater than 0.1.

HPV-16 VLP ELISA. (i) Coating antigen. ELISAs were carried out with VLPs containing the HPV-16 L1 sequence variant corresponding to our virus stock. DNA was extracted from the second-passage HPV-16 virus stock as described above. The entire L1 gene was amplified by PCR and cloned into a baculovirus transfer vector by methods similar to those previously described (31). The DNA sequence of the full L1 clone was determined. The sequence was identical to that of the MY09/MY011 amplicon in the overlapping region and contained 11 additional nucleotide substitutions outside the region amplified by the MY09/MY011 primer pair. In total, this HPV-16 L1 variant contained nine amino acid differences from the prototype: histidine to tyrosine (C5862T), threonine to asparagine (C6163A), asparagine to threonine (A6178C), histidine to aspartic acid (C6240G), glycine to serine (G6252A), threonine to alanine (A6432G), threonine to proline (A6693C), threonine to serine (A6801T), and leucine to phenylalanine (G7058T). The transfer vector containing the HPV-16 L1 gene was used to generate a recombinant baculovirus as previously described (31). VLPs were purified from recombinant baculovirus-infected High Five cells as described above.

(ii) ELISA protocol. HPV-16 L1 VLPs were diluted in PBS to 0.01 mg/ml and dispensed in 0.1-ml aliquots to 96-well microtiter plates. PBS without VLPs was dispensed to control wells. After being coated for 16 h at 4°C, the plates were blocked with blocking solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 2 h at room temperature. Threefold serial dilutions of anti-HPV-VLP sera were made in PBS containing 1% bovine serum albumin and 10% (vol/vol) wild-type baculovirus-infected cell culture supernatant to reduce the background (30). After a 90-min room temperature incubation, plates were washed and anti-rabbit immunoglobulin G-alkaline phosphate conjugate (Kirkegaard & Perry Laboratories, Inc.) diluted 1:2,000 in blocking solution was added to the wells. Following incubation and washing, specific binding was detected with the alkaline phosphate substrate. Specific absorbance was calculated by subtracting the absorbance values obtained with PBS alone from those obtained with antigen. Averages of duplicate wells were calculated as the final absorbance values.

HPV-16 in vitro infection and neutralization. HaCaT cells, an immortalized human keratinocyte cell line (6), were kindly supplied by N. Fusenig. Cells were grown to 90 to 100% confluency in 154/HKGS medium (Cascade Biologics, Inc., Portland, Oreg.) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) in 24-well plates. HPV-16 stock was sonicated for 25 s on ice, diluted into 154/HKGS medium in round-bottom polypropylene tubes, and incubated for 1 h at 37°C. Medium was aspirated from the HaCaT cells, and 0.5 ml of diluted virus was added per well. As a control, one well of cells on each plate received 0.5 ml of medium without virus. For antibody-mediated neutralization, antisera were diluted in 154/HKGS and incubated with a fixed quantity of the HPV-16 stock in a final volume of 0.5 ml in round-bottom polypropylene tubes fo r 1 h at 37°C prior to addition to the HaCaT cells. Fresh medium was added to each well of cells 4 days postinfection, and on day 7, total cellular RNA was extracted with Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacturer's recommendations. Final RNA pellets were resuspended in 20 μl of diethylpyrocarbonate-treated water and quantified by spectrophotometry.

Detection of HPV-16 and cellular β-actin-spliced mRNA by RT-PCR. Reverse transcription reactions were performed with a First Strand cDNA kit (Boehringer Mannheim, Indianapolis, Ind.) with 2 μg of total RNA as the template and oligo(dT) as the primer in a final volume of 20 μl. Nested PCR was needed to detect HPV-16 E1 ^ E4 cDNA. The first round of amplification was carried out with 25% of the cDNA from each reverse transcription reaction and 5 '-TGGA AGACCTGTTAATGGGCACAC-3 ' (located at bases 797 to 818 in the HPV-16 genomic sequence) as the forward outside (FO) primer and 5 '-TATAGACAT AAATCCAGTAGACAC-3 ' (located at bases 3826 to 3849 in the HPV-16 genomic sequence) as the reverse outside (RO) primer for 40 cycles of PCR. Ten percent of the first-round PCR mixture was used for nested reactions with 5'-GGAATTGTGTGCCCCATCTGTTC-3 ' (located at bases 823 to 845 in the HPV 16 genomic sequence) as the forward nested primer (FN) and 5 '-GTTCA CGTTGACATTCACTATC-3 ' (located at bases 3766 to 3787 in the genomic sequence) as the reverse nested primer (RN) for 35 PCR cycles. First-round and nested PCRs were set up with Hot Wax beads (1.5 mM) and pH 9.5 buffer (InVitrogen, San Diego, Calif.) with 200 μM deoxynucleoside triphosphates (dNTPs), 125 ng each of the forward and reverse primers, and 2.5 U of *Taq* polymerase (Perkin-Elmer, Foster City, Calif.) in a final volume of 50 μl. The temperature profile for both first-round and nested PCRs was 80°C for 5 min, 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

All cDNA samples were used in separate PCRs with primers specific for spliced cellular β-actin mRNA. The PCR primers were described by Smith et al. (34). Amplification of the β-actin spliced message was achieved with 125 ng of forward primer (5'-GATGACCCAGATCATGTTTG-3 ') and 125 ng of reverse primer (5'-GGAGCAATGATCTTGATCTTC-3 ') with 12.5% of the total cDNA as the template in 10 mM Tris-HCl (pH 8.3) buffer containing 50 mM KCl, 1.5 mM $MgCl_2$, and 1% gelatin, with 200 μM dNTPs and 2.5 U of *Taq* polymerase in a final volume of 50 μl for 35 PCR cycles. The temperature profile for amplification was 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

All PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide fluorescence.

DNA sequencing. The E1 ^ E4 nested PCR product was purified with Qiaquick-spin columns (Qiagen, Chatsworth, Calif.). The concentration of the column eluate was determined by spectrophotometry and was then diluted to achieve a final concentration of 125 ng/μl. Sequencing reactions were carried out with the Dye Terminator DNA sequencing reaction mix (Perkin-Elmer, Foster City, Calif.) with 125 ng of PCR product as template and 3 pmol of FN or RN primers. Samples were subjected to cycle sequencing according to the manufacturer's recommendation. Extension products were purified with Centri Sep columns (Princeton Separations, Adelphia, N.J.) and dried under vacuum. Samples were resuspended in 4 μl of sequencing buffer (formamide containing 8.3 mM

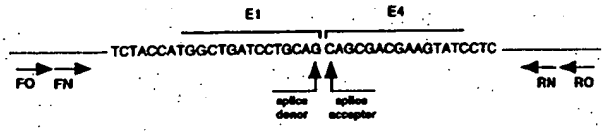

FIG. 1. RT-PCR strategy for the detection of HPV-16 E1^E4 mRNA. Total cellular RNA obtained from HPV-16-exposed HaCaT cells was reverse transcribed, and the resultant cDNA was amplified by PCR with the FO and RO primers as described in Materials and Methods. Nested PCR with the FN and RN primers resulted in the amplification of a 487-bp product. Nucleotide sequence analysis of the PCR product revealed the splice donor (nucleotide 881)/acceptor (nucleotide 3356) site of HPV-16 E1^E4 mRNA.

EDTA) and electrophoresed on a 4.2% acrylamide–8 M urea sequencing gel with the ABI 373 automated sequencer (ABI, Foster City, Calif.). Sequence data were analyzed with the Laser Gene program (DNA Star, Madison, Wis.).

RESULTS

HPV-16 infects human keratinocytes in vitro. An HPV-16 stock ($HPV\text{-}16_{Rochester\text{-}1K/ur3}$) that represented the third passage in xenografts of a viral lysate originally derived from patients with a history of condylomata acuminata was used to infect the human keratinocyte cell line HaCaT. Since HPV-16 was not expected to progress through an infectious cycle in cultured cells, an RT-PCR strategy was designed to detect an HPV-16-specific E1^E4 mRNA as a marker for infection (Fig. 1). E1^E4 mRNA species have been demonstrated to be very abundant in HPV-1-, HPV-6-, and HPV-11-induced condylomas (8, 9, 24, 25), as well as in an HPV-16-transformed rodent cell line (36, 37). In addition, an HPV-11-specific E1^E4 transcript was successfully used as a direct marker for HPV-11 infection in both the xenograft system and in cultured human cells (4, 33, 34).

HPV-16 stock was diluted and added to cultured HaCaT cells. After 7 days in culture, total RNA was extracted from the cells and was used for cDNA synthesis. Nested primers were then used to amplify an HPV-16 E1^E4 cDNA. A 487-bp PCR product consistent with the projected size of an HPV-16 E1^E4 transcript was amplified from the RNA of the cells incubated with virus (Fig. 2, lane 1). In contrast, no similar PCR product was detected with RNA isolated from control HaCaT cells which had not been exposed to virus (Fig. 2, lane 2). The inability to amplify the 487-bp product from the control cellular RNA was not due to poor RNA recovery or failed reverse transcription, since the cDNA sample was successfully used in a separate PCR to detect spliced β-actin mRNA (Fig. 2, lane 2).

The identity of the 487-bp HPV-16-specific PCR product was confirmed by nucleotide sequence analysis. The DNA sequence of this PCR product represented HPV-16 nucleotides 823 to 3787, with a deletion spanning nucleotides 881 to 3356, consistent with an HPV-16 E1^E4 spliced mRNA species (Fig. 1) (32).

The results presented in Fig. 2 were obtained with the HPV-16 virus stock diluted $1:10^4$. However, identical results were obtained with dilutions of virus stock from $1:10^2$ to $1:10^6$. The 487-bp HPV-16 product was not amplified from cells which had been exposed to the virus stock diluted to $1:10^7$ (data not shown). Repetitive titration of the viral stock resulted in consistent detection of the HPV-16 spliced message with viral dilutions of $\leq 1:10^6$.

Neutralization of HPV-16 in vitro infection. No cytopathic changes were associated with HPV-16 infection of HaCaT cells. However, the ability to detect an HPV-16-specific mRNA following exposure of HaCaT cells to the viral stock indicated

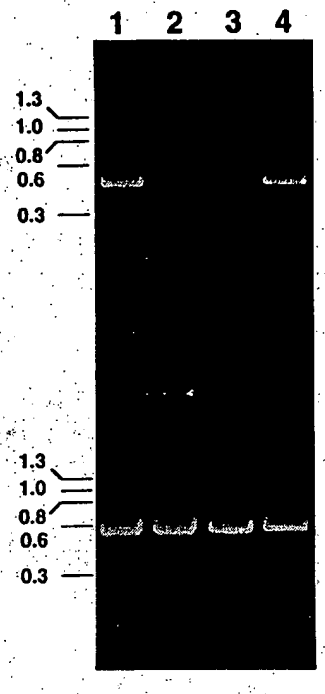

FIG. 2. HPV-16 infection of HaCaT cells documented by the appearance of an HPV-16-specific E1^E4 mRNA. cDNA samples obtained by reverse transcription of total RNA isolated from cells infected with HPV-16 (lane 1), uninfected cells (lane 2), cells infected with HPV-16 preincubated with HPV-16 L1 VLP antiserum (lane 3), and cells infected with HPV-16 preincubated with normal control serum (lane 4) were amplified with primers specific for HPV-16 E1^E4 (top) or β-actin (bottom).

that the virus entered the cells and began its replication cycle at least to the point of expression of an E1^E4 transcript. To determine if specific antibodies could neutralize HPV-16 infection, the virus stock was preincubated with polyclonal anti-HPV-16 L1 VLP serum or normal control serum prior to addition to the HaCaT cells. Virus neutralization was demonstrated by the inability to detect the E1^E4 spliced message in virus-exposed cells following preincubation of the virus stock with a 1:100 dilution of the anti-HPV-16 L1 VLP serum (Fig. 2, lane 3). In contrast, the HPV-16 transcript was detected in cells incubated with virus mixed with control serum (Fig. 2, lane 4).

Polyclonal antisera were also generated against L1 VLPs corresponding to certain low-risk (types 6 and 11) and high-risk (types 18, 31, 33, 35, 39, 45) HPVs and were screened by ELISA against homotypic VLPs. Each of the antisera reacted strongly with homotypic VLPs with titers of $\geq$1:121,500. This panel of anti-VLP sera was tested for HPV-16-neutralizing activity by preincubation of a 1:100 dilution of each serum sample with the HPV-16 stock prior to exposure to the cells. As shown in Fig. 3, none of the heterotypic VLP antisera inhibited HPV-16 infection, except anti-HPV-33 L1 VLP. Additional experiments conducted with lower (1:20) dilutions of antisera confirmed that the HPV-6, -11, -18, -31, -35, -39, and -45-specific antibodies were unable to neutralize HPV-16.

A quantitative assessment of the relative potency of HPV-16-neutralizing activity in the anti-HPV-16 and anti-HPV-33 VLP antisera was carried out by incubation of the HPV-16 stock with serial $\log_{10}$ dilutions of the serum samples. The anti-HPV-16 VLP serum inhibited the detection of the

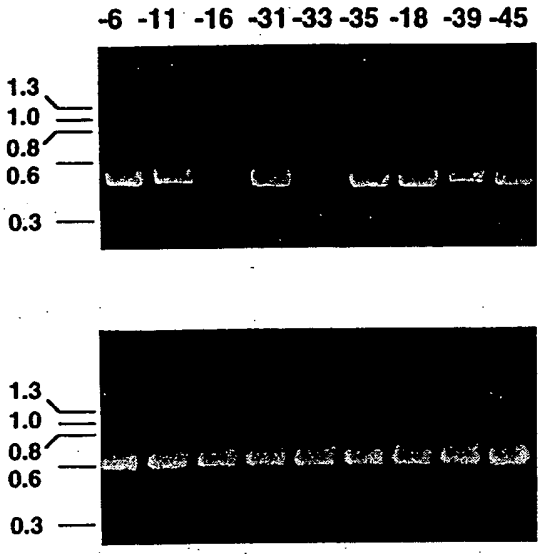

FIG. 3. HPV-16 neutralization by heterotypic genital HPV L1 VLP antisera. HPV-16 stock diluted $1:10^4$ was preincubated with 1:100 dilutions of anti-HPV L1 VLP serum samples prior to addition to HaCaT cells. HPV-16 E1^E4 (top)- and cellular β-actin (bottom)-specific RT-PCR products obtained with RNA isolated from cells infected with HPV-16, which had been preincubated with anti-HPV-6, -11, -16, -31, -33, -35, -18, -39, and -45 L1 VLP sera, are shown.

E1^E4 mRNA at dilutions of $\leq 1:10^5$, while the anti-HPV-33 VLP serum only neutralized HPV-16 at dilutions of $\leq 1:10^3$ (Fig. 4).

The differential neutralizing activity of the anti-HPV-16 VLP serum and the anti-HPV-33 VLP serum against HPV-16 was reflected in the relative ability of the two antisera to bind HPV-16 VLPs in an ELISA (Fig. 5). The anti-HPV-33 VLP serum exhibited binding to HPV-16 L1 VLPs, although to a much lesser extent than the homotypic anti-HPV-16 VLP serum. All of the remaining heterotypic antisera reacted very weakly with HPV-16 VLPs. Thus, with this panel of antisera, the relative ability to neutralize HPV-16 correlated with binding to HPV-16 L1 VLPs.

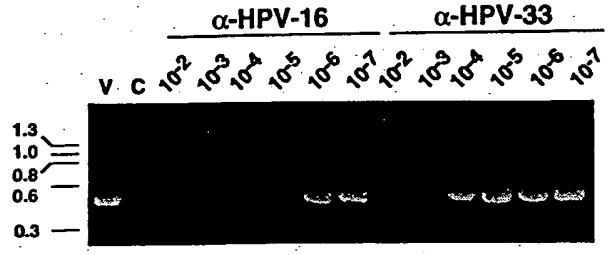

FIG. 4. Titration of the HPV-16-neutralizing activity in the anti (α)-HPV-16 VLP and the anti-HPV-33 VLP sera. HPV-16 stock diluted $1:10^4$ was preincubated with serial $\log_{10}$ dilutions of anti-HPV-16 L1 VLP or anti-HPV-33 L1 VLP antiserum prior to addition to HaCaT cells. RT-PCR products obtained with HPV-16-specific primers are shown. Lanes are labeled with the reciprocal dilution of antiserum used in the experiment. Lane V represents RT-PCR product obtained with RNA isolated from cells infected with HPV-16 preincubated with a 1:100 dilution of normal serum. Lane C represents the RT-PCR product obtained with RNA from uninfected cells.

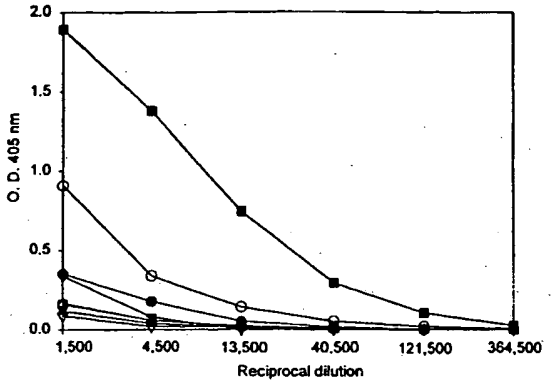

FIG. 5. Reactivity of the anti-HPV VLP sera against HPV-16 L1 VLPs. ELISA plates were coated with HPV-16 VLPs and incubated with threefold serial dilutions of antisera to the following VLP types: HPV-11 (●), HPV-16 (■), HPV-18 (▲), HPV-31 (▼), HPV-33 (○), HPV-35 (□), HPV-39 (△), and HPV-45 (▽). Specific binding was detected with an anti-rabbit immunoglobulin G secondary antibody reagent as described in Materials and Methods. O.D., optical density.

DISCUSSION

We have developed an RT-PCR-based in vitro assay which can be used to study the early stages of HPV-16 infection and to examine antibody-mediated virus neutralization. The assay is relatively rapid and highly reproducible, and thus it is amenable to the evaluation of large numbers of serum samples for HPV-16-neutralizing antibodies. Since the assay is semiquantitative, it can be used to derive end point titers of HPV-16-neutralizing antibodies. The sensitivity of RT-PCR allows the HPV-16 stock to be used at relatively high dilutions ($1:10^4$ to $1:10^6$), thereby conserving the virus, which is in limited supply. The amount of virus utilized in the assay is described as a viral stock dilution, since there is no methodology for determining the number of infectious HPV-16 particles. It is anticipated that a different viral stock would contain a different number of infectious particles and would require titration to maintain assay reproducibility.

The appearance of the spliced HPV-16-specific transcript in cells which had been cultured with the virus indicated that the initial stages of infection had been successfully accomplished: cell attachment and entry, uncoating, translocation, and transcription. This is an important consideration in studies of antibody-mediated virus neutralization, since it is not known which steps in the infection process are inhibited by antibodies. Roden et al. found that monoclonal antibodies to BPV-1 could neutralize BPV-1 either by inhibiting cell binding or by blocking a subsequent step in the infection pathway (29). Christensen et al. also reported antibody neutralization of BPV-1, CRPV, and HPV-11 at a step in the infectious cycle subsequent to virus attachment (10). Thus, the RT-PCR-based in vitro infectivity assay for HPV-16 represents a significant improvement over surrogate assays, such as VLP-mediated hemagglutination inhibition, which only detect antibodies that inhibit cell attachment and therefore may underestimate the virus-neutralizing potential of an antiserum sample (28).

Using the RT-PCR-based assay, we demonstrated that HPV-16 was neutralized by antibodies raised against HPV-16 L1 VLPs. This result was anticipated, since HPV-16 L1 VLPs had previously been shown to elicit production of antibodies which inhibited both HPV-16 L1 VLP-mediated hemagglutination and infection of cultured cells by HPV-16 pseudovirions

(27, 28). However, neutralization of authentic HPV-16 virions lends further support to the potential application of VLPs as prophylactic HPV vaccines.

Whereas HPV-16 is the most prevalent high-risk HPV type, broad protection against cervical cancer would require that a vaccine target multiple HPV types (e.g., HPV-16, -18, -31, -33, and -45). Evaluation of the ability of different HPV VLPs to elicit production of cross-neutralizing antibodies is important in determining the ultimate composition of a broadly efficacious vaccine. The HPV-16 in vitro infectivity assay was used to test antisera against heterotypic HPV L1 VLPs for neutralizing activity against the virus. Antisera to the low-risk HPV-6 and -11 and the high-risk HPV-18, -31, -35, -39, and -45, each containing high titers of homotypic antibodies as measured by ELISA, all failed to neutralize HPV-16 infection. This result is in agreement with previous findings which demonstrated that antibody responses to the genital HPVs are largely type specific (27, 28, 30). In this regard, our observation that anti-HPV-33 L1 VLP antibodies neutralized HPV-16 is somewhat surprising. Previously, cross-neutralization had only been observed consistently with very closely related virus types, such as HPV-6 and -11, which possess L1 amino acid sequence identity of >90% (11). HPV-16 neutralization by antiserum raised against HPV-33 VLPs was not suggested by in vitro infectivity assays with HPV-16 pseudovirions (27). Weak cross-neutralization between HPV-16 and HPV-33 was seen in some hemagglutination assays with HPV-33 and -16 VLPs but was not consistently observed, thereby suggesting that detection of potential neutralizing activity was below the sensitivity of the assay (28). In contrast, by the RT-PCR-based assay, HPV-16 neutralization by anti-HPV-33 VLP antiserum was reproducible and titratable. Thus, the difference between our current result and previous results obtained by surrogate neutralization assays may indeed relate to differences in assay sensitivity. Alternatively, the discrepancy could be attributable to differences in the quantity and/or quality of the anti-HPV-33 VLP antibodies used in the different assays.

The amino acid sequence of HPV-16 L1 shares greater homology with HPV-31 L1 and HPV-35 L1 than with HPV-33 L1 (83.1, 82.8, and 79.7% amino acid sequence identity, respectively). However, amino acid sequence identity may not correlate strictly with structural similarity. It is well established that neutralizing antibodies raised against intact authentic HPV capsids and recombinant papillomavirus VLPs primarily recognize conformation-dependent epitopes on viral particles (12–15). Thus, the HPV-16-neutralizing capability of the anti-HPV-33 antibodies may reflect a greater degree of structural similarity between HPV-16 and HPV-33 than might be predicted by amino acid sequence comparisons.

Our current results suggest that HPV-33 and HPV-16 share a neutralizing epitope or epitopes. However, due to the lack of an HPV-33 stock, direct evaluation of the neutralizing activity of the HPV-33 and -16 L1 VLP antisera against HPV-33 was not possible. The anti-HPV-33 L1 VLP serum was not as potent as the anti-HPV-16 L1 VLP antiserum at neutralizing HPV-16, suggesting that HPV-33 and HPV-16 may possess both common and distinct neutralization sites similar to those previously reported for HPV-6 and HPV-11 (11). The ability of the anti-HPV-33 VLP serum to neutralize HPV-16 correlated with its ability to bind HPV-16 L1 VLPs in an ELISA. Interestingly, when the anti-HPV-16 L1 VLP serum was assayed for binding to HPV-33 L1 VLPs, no reactivity was detected (data not shown). Unckell et al. have reported neutralization of HPV-33 pseudovirions with an anti-HPV-33 VLP serum but not with an anti-HPV-16 VLP serum (38). Complete assessment of cross-neutralizing activities among the different genital HPV types will require the generation of additional infectious viral stocks and the development of the respective quantitative infectivity assays.

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Expression of the Myelomonocytic Antigens CD36 and L1 by Keratinocytes in Squamous Intraepithelial Lesions of the Cervix

NICHOLAS COLEMAN, MBChB, AND MARGARET A. STANLEY, PhD

The keratinocytes in squamous intraepithelial lesions (SILs) of the cervix show altered expression of a number of molecules involved both in the control of growth and differentiation and in cell surface interactions, particularly with components of the immune system. We have used tissue biopsies and in vitro model systems to investigate the expression in SILs of the molecules CD36 and L1, which are predominantly expressed by myelomonocytic cells but which also have functional roles in keratinocyte biology. Whereas the L1 protein (defined by the monoclonal antibody Mac387) was expressed by suprabasal and superficial cells in 12 of 12 cases of normal cervix (NCx) and in 14 of 14 cases of low-grade SILs (LG-SILs), in two of 16 cases of high-grade SILs (HG-SILs) it was entirely absent and in the remainder it was restricted to the most superficial layers. When an arbitrary grading scale was applied, L1 expression in HG-SILs proved to be significantly lower than in LG-SILs ($P < .01$) or in cases of NCx ($P < .01$). CD36 was expressed by superficial cells in four of 12 cases of NCx, in six of 14 LG-SILs, and none of 16 cases of HG-SILs (when graded, LG-SILs *v* HG-SILs = $P < .05$). The mechanisms underlying the expression of both molecules were investigated by growth in organotypic tissue culture of normal ectocervical epithelium and the cervical keratinocyte cell lines W12 (a model for LG-SILs) and CaSki and SiHa (models for HG-SILs). L1 was diffusely expressed by NCx cells and the W12 cell line, although its expression in the CaSki and SiHa cell lines was much more irregular and restricted. CD36 was occasionally present on the surface of superficial NCx and W12 cells, but was absent from CaSki and SiHa cells. Neither molecule could be induced by treatment of the cells with interferon-gamma. These data suggest that the expression of CD36 and L1 by cervical keratinocytes is related to their differentiation status rather than representing an effect of exogenous factors, such as those released by the immune cell infiltrate associated with SILs. CD36 may function as an immunoregulatory molecule on cervical keratinocytes in SILs, while L1 is more likely to be involved in the intracellular regulation of cell proliferation and maturation. HUM PATHOL 25:73–79. Copyright © 1994 by W.B. Saunders Company

The human papillomaviruses (HPVs) have been strongly implicated in the development of squamous cell carcinoma of the uterine cervix[1] and of the precursor squamous intraepithelial lesions (SILs), which may be of low or high grade according to the degree of proliferative activity and loss of differentiation of the epithelial cells.[2] The fate of ectocervical epithelium after infection with HPV appears to depend not only on intracellular events controlling the capacity for proliferation and differentiation,[3] but also on extracellular interactions, particularly with components of the immune system.[4] There have been several reports of alterations in the numbers of immune cells in cervical disease, including Langerhans' cells,[5] lymphocytes,[6] and macrophages,[7] although the mechanisms for these changes are not known. It is becoming increasingly clear, however, that the neoplastic ectocervical keratinocytes may play an important role in modulating the local immune cell repertoire. These cells show altered expression of a number of immunocompetent molecules, including MHC class I,[8] MHC class II,[9] and adhesion molecules such as ICAM1.[10]

The molecules L1 and CD36 are predominantly expressed by leukocytes but also are potentially of importance in the intracellular and extracellular functioning of keratinocytes. There has been considerable interest in the expression of these molecules by squamous epithelium in a variety of sites and several studies have documented altered expression by keratinocytes in a number of pathologic conditions. CD36 is a multifunctional molecule predominantly expressed by platelets, monocytes, macrophages, and some endothelial cells.[11,12] It acts as a receptor for the extracellular matrix protein thrombospondin,[13] has a role in signal transduction, and is potentially important in certain immune interactions, particularly those involving immunologic accessory cells.[14] The L1 antigen, referred to inter alia as calprotectin,[15] is predominantly expressed by monocytes and neutrophils in the peripheral blood[16] and in the tissues by eosinophils and a subset of reactive macrophages.[17] As well as being implicated as a component of the innate defence system, there is evidence that the molecule also may serve to regulate epithelial cell proliferation and differentiation (reviewed by Brandtzaeg et al[15]). We report the expression of CD36 and L1 by keratinocytes in cases of normal and diseased cervix. We have observed alterations in the patterns of expression of both molecules in high-grade SILs (HG-SILs) and have used in vitro model systems to explore the mechanisms by which these changes arise.

MATERIALS AND METHODS

Specimens

Cervical punch biopsy specimens were taken from patients with abnormal colposcopic appearances and/or recent evidence of abnormal cervical cytology. Age-matched females undergoing laparoscopic sterilization were used as controls; these women showed no evidence of ongoing or previous cervical disease. All biopsy specimens were immediately mounted in OCT compound (BDH Ltd, Poole, UK) and snap-frozen in liquid nitrogen before being stored for up to 6 months in

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Address correspondence and reprint requests to Nicholas Coleman, Department of Pathology, University of Cambridge, Tennis Court Rd, Cambridge, CB2 1QP, UK.

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liquid nitrogen prior to use. Serial 6-μm frozen sections were cut, and routine haematoxylin-eosin staining was performed on every eighth level. On the basis of the hematoxylin-eosin staining the biopsy specimens were classified as histologically normal (n = 12) or as representing low-grade SILs (LG-SILs) (n = 14) or HG-SILs (n = 16). The morphologic changes were equivalent to those of koilocytosis and/or CIN1 in the LG-SILs and to CIN2 and CIN3 in the HG-SILs. After morphologic evaluation the remainder of the tissue cut from each section underwent immunohistologic assessment.

Immunohistology

The primary monoclonal antibodies used were OKM5 (Ortho Ltd; mouse IgG1, diluted 1:30) and SM0 (Serotec Ltd; mouse IgM, diluted 1:20) against CD36, and Mac387 (Dako Ltd; mouse IgG1, diluted 1:200) against L1. Each of the anti-CD36 antibodies gave identical staining results. Immunoperoxidase staining was performed using standard protocols, with avidin-biotin amplification and visualization of peroxidase activity with diaminobenzidine.

We devised an arbitrary grading scale to obtain a semiquantitative representation of antigen expression in the biopsy specimens. A total score was awarded for each tissue section, which represented the sum of the intensity and the extent of staining. The two parameters were scored as follows:

- Intensity: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining.
- Distribution: 0, occasional positivity; 1, patchy positivity; and 2, widespread positivity.

The results from each group of biopsy specimens were compared using the Wilcoxon rank sum test. Probability values, when given, are two-sided.

Cell Culture

Normal cervical epithelium (NCx) was cultured from uteri removed for noncervical disease. All patients had recently documented normal cervical smears and sections of uncultured cervical tissue appeared normal histologically. Cell culture was performed using protocols previously described.[18] Briefly, a sheet of ectocervical epithelium, which included the squamocolumnar junction, was finely minced before being stirred for 30 minutes at 37°C in a solution of 0.25% trypsin/0.01% EDTA in phosphate-buffered saline (PBS) to produce a single-cell suspension. The cells were grown with support from lethally irradiated Swiss G3T3 mouse fibroblast feeder cells and were maintained in Glasgow's modification of Eagle's medium (GMEM), supplemented with 10% (vol/vol) fetal calf serum (SeraLabs Ltd), 0.1 μg/mL hydrocortisone, and 10^{-10} mol/L cholera toxin (both Sigma Ltd). Colony formation was established by adding epidermal growth factor (Sigma Ltd) at 10 ng/mL 24 hours after plating. At subconfluence the feeders were removed with 0.01% EDTA in PBS and the keratinocytes were dislodged with 0.1% trypsin/0.01% EDTA in PBS before seeding onto collagen gels to establish them in organotypic culture (see below).

The cervical keratinocyte cell line W12 was derived from a cervical wart and contains HPV 16 DNA, predominantly in the episomal form, with approximately 100 copies/cell.[19] The cells are nontumorigenic in nude mice and can reform an epithelium that resembles LG-SILs. The W12 cell line is a unique in vitro model for the early stages of HPV 16-related cervical disease. The cells were grown with Swiss G3T3 support using protocols identical to those described for NCx cells before being established in organotypic culture.

The SiHa[20] and CaSki[21] cell lines were each derived from a squamous cell carcinoma of the cervix. Each is a tumorigenic cervical keratinocyte line containing integrated HPV 16 DNA, with one and 300 to 500 copies/cell, respectively. Prior to organotypic culture the cells were grown without feeder support in GMEM supplemented with 10% (vol/vol) fetal calf serum (SeraLabs Ltd).

Organotypic "Raft" Culture

The "raft" system of organotypic tissue culture allows cells growing on a collagen "gel" to differentiate at an air to liquid interface and enables them to produce stratified epithelia resembling the original lesions from which they were established. Eight milliliters of Vitrogen 100 collagen (Collagen Corp Ltd) were mixed on ice with 1.0 mL of 10× Vitrogen PBS solution and 1.0 mL of 0.1 mol/L NaOH. Two milliliters of the gel solution were then layered onto 5×10^5 lethally irradiated Swiss G3T3 fibroblasts in a 35-mm tissue culture dish, and gelation was initiated by warming to 37°C for 2 hours followed by equilibration with 2.0 mL of GMEM at 37°C overnight. The next day 10^6 keratinocytes were applied to the surface of each gel and maintained until confluent in GMEM supplemented with 10% fetal calf serum and cholera toxin. The collagen was then raised onto a metal grid such that the cells were able to differentiate at an air to liquid interface. After a further 10 days the collagen "rafts" were removed and cut into strips, which were mounted in OCT compound and snap-frozen in liquid nitrogen. Six-micrometer frozen sections were cut and immunohistochemical analysis was performed as described for the tissue biopsy specimens.

RESULTS

Staining of Tissue Biopsy Specimens

Normal Cervix. In all biopsy specimens from normal ectocervix the keratinocytes showed cytoplasmic expression of the L1 protein (Table 1). This was present in the suprabasal and superficial layers, with sparing of the basal cells (Fig 1, top left). CD36 was expressed on the surface of keratinocytes in four of the 12 specimens examined. The staining was generally patchy and weak, and was most prominent in the superficial layers, where it produced a net-like pattern (Fig 1, bottom left), although some basal positivity also was observed occasionally. There was no spatial association between CD36+ keratinocytes and infiltrating immune cells.

Low-grade Squamous Intraepithelial Lesions. L1 expression was seen in the cytoplasm of keratinocytes in all 14 of the LG-SILs examined. Its distribution within the epithelium was similar to that in normal ectocervix and included expression by koilocytes (Fig 1, top center). In six of the 14 biopsy specimens keratinocytes showed surface expression of CD36 (Fig 1, bottom center). In some specimens the expression was patchy and weak, similar to that in normal ectocervix, although in others it was stronger and more extensive. Again, there was no correlation between CD36+ keratinocytes and infiltrating immune cells.

High-grade Squamous Intraepithelial Lesions. Reduced expression of L1 was seen in HG-SILs. The molecule could not be detected in two of the 16 biopsy specimens examined and in the majority of the remainder it was restricted to the most superficial layers (Fig 1, top right). In general, L1 was detectable in areas of cellular differentiation and was absent from the mor-

TABLE 1. Semiquantitative Representation of CD36 and L1 Expression in Cervical Biopsy Specimens

Biopsy	Antigen					
	CD36			L1		
	NCx	LG-SILs	HG-SILs	NCx	LG-SILs	HG-SILs
No. of biopsy specimens examined	12	14	16	12	14	16
No. of positive biopsy specimens	4	6	0	12	14	14
Mean score	0.42‡	0.71*	0	4.00†	3.79†	2.81
Median score	0	0	0	4	4	2
Range of scores	0-2	0-3	0	3-5	2-5	0-4

Note: An arbitrary grading scale was used (see Materials and Methods section). *Statistical analysis:* Differences between high-grade lesions and low-grade lesions or normal cervix. * $P < .05$; † $P < .01$; ‡not significant. There was no significant difference between low-grade lesions and normal cervix for either molecule (Wilcoxon rank sum test; P values are two-sided).

phologically most atypical cells. There was no expression of CD36 by keratinocytes in any of the HG-SILs (Fig 1, bottom right).

An arbitrary grading scale was used to quantitate the expression of CD36 and L1 by cervical keratinocytes in each of the three groups (Table 1). The expression of CD36 in the LG-SILs was significantly greater than that in the HG-SILs ($P < .05$), although there was no significant difference between the LG-SILs or HG-SILs, and cases of NCx. L1 expression in HG-SILs was significantly less than that in the LG-SILs ($P < .01$) or cases of NCx ($P < .01$), but there was no significant difference in L1 expression between the LG-SILs and cases of NCx.

Staining of Cell Lines

The mechanisms underlying the expression of CD36 and L1 in vivo were explored using cell culture techniques. When the cervical keratinocytes were grown in monolayer culture no expression of CD36 and L1 (as determined by flow cytometry) was observed in any case (data not shown). Cell stratification and maturation were induced using the "raft" technique of organotypic tissue culture, and the cytokine-mediated inducibility of the molecules on each cell type was quantified by culturing the cells in the presence of recombinant interferon-gamma.

Normal cervical cells produced a differentiating epithelium up to 10 cell layers thick. L1 was expressed by all cells except those in the basal layers, and was cytoplasmic in distribution (Fig 2, top left). CD36 was expressed infrequently and only by superficial and differentiated cells (arrowed cell, Fig 2, top right).

W12 produced a somewhat thinner epithelium showing disorganization of the basal layer but with surface differentiation consistent with a low-grade cervical lesion. There was strong cytoplasmic expression of L1 by the suprabasal and superficial cells (Fig 2, center left). Basal cells were generally negative, although there was some focal expression. CD36 was expressed on the surface of superficial, differentiated cells (Fig 2, center right), including some that were vacuolated and that resembled kollocytes.

CaSki cells produced an epithelial layer of up to 12 cells. This appeared disorganized and highly atypical throughout its full thickness, reminiscent of a high-grade cervical lesion. There was mild irregularity of the base of the epithelium, but no unequivocal infiltration of the subjacent collagen. L1 showed a restricted pattern of expression, with many areas entirely negative for the molecule. Patchy positivity was observed in some places, however, and this occurred throughout the full thickness of the epithelium (Fig 2, bottom left). CD36 was not seen in any of the sections examined (Fig 2, bottom right). SiHa cells also produced an atypical epithelial layer, and showed patterns of expression of both molecules that were essentially the same as those seen on CaSki cells (data not shown).

All cell types were grown in organotypic culture in the presence of recombinant interferon-gamma. Different doses (150 and 300 U/mL) were used, and culture was maintained for 24, 48, and 72 hours prior to sectioning. However, no detectable modulation of either molecule was seen on any of the four cell types examined.

DISCUSSION

The mechanisms underlying the development of intraepithelial neoplasia in the ectocervix are complex. The abnormal keratinocytes show a number of alterations in molecules likely to be involved in intracellular signalling and control of proliferation[5,22,23] as well as in interactions with cells and molecules in the extracellular environment.[8,9,24] CD36 and L1 have important roles in leukocyte biology[13-15] and may contribute to intracellular and extracellular interactions of keratinocytes at a number of sites.[25-27] The expression and modulation of these molecules in the cervix have not been investigated previously, however.

The distribution of CD36 in cases of NCx is consistent with some reports of its localization in the skin,[27,28] although the factors controlling expression of the molecule by cervical keratinocytes are unclear. Whereas interferon-gamma has been shown to upregulate CD36 on normal epidermal keratinocytes in vitro,[29] an observation supported by evidence of increased CD36 expression on keratinocytes in inflamed skin,[30,31] this cytokine is unlikely to contribute significantly to the expression seen in the cervix. We saw no association

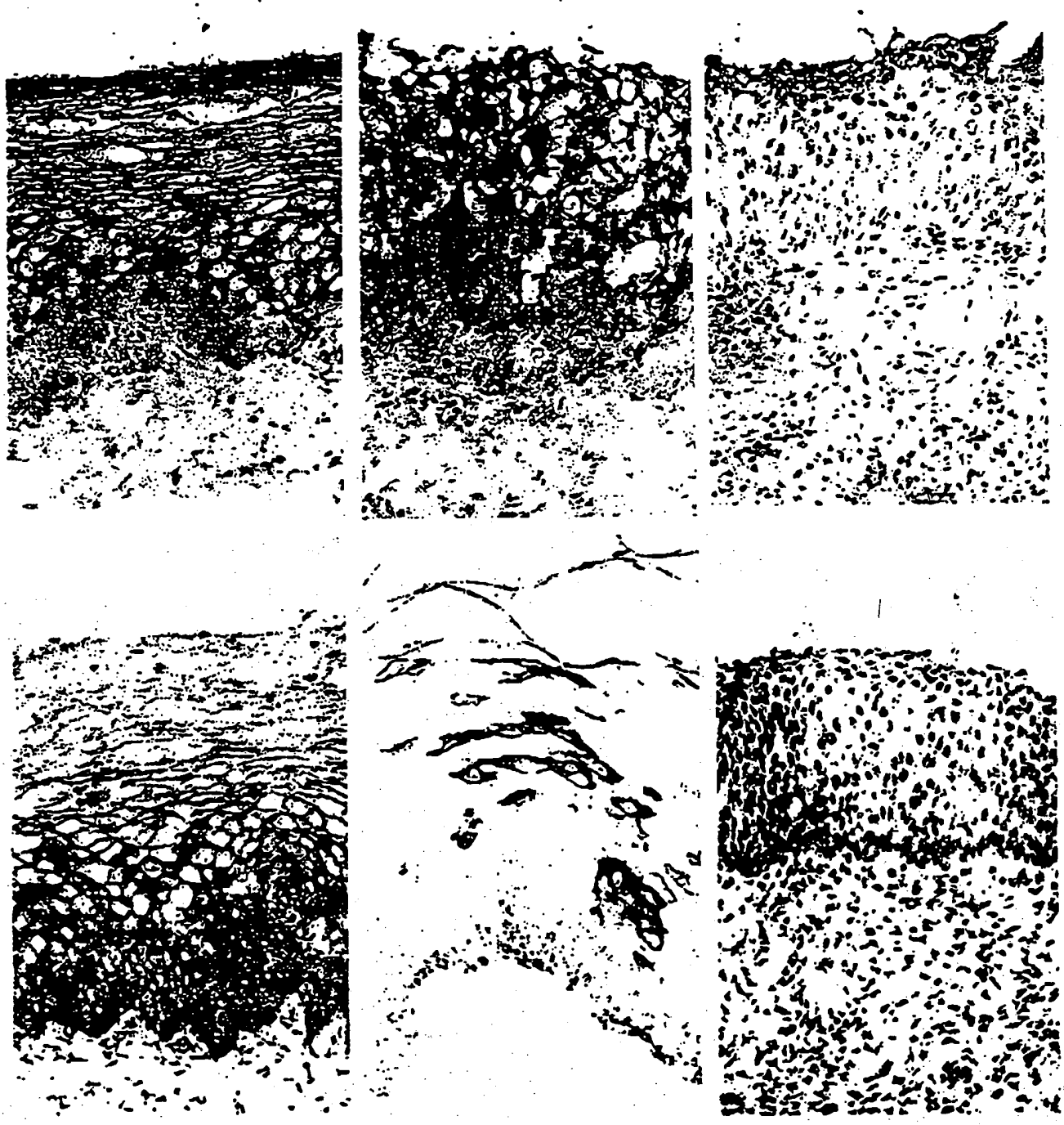

FIGURE 1. Expression of L1 (top) and CD36 (bottom) on keratinocytes in cases of NCx (top left and bottom left), LG-SILs (top center and bottom center), and HG-SILs (top right and bottom right). See text for details. (Immunoperoxidase staining using avidin-biotin amplification and diaminobenzidine as chromogen; magnifications ×160.)

between CD36 positivity and numbers of lymphocytes in the epithelium or subepithelial stroma of any of the cervical biopsy specimens we examined, and recombinant interferon-gamma did not modulate CD36 expression on the cervical keratinocytes in tissue culture. It is more likely that the presence of CD36 is related to the maturational state of the cervical keratinocytes, as has been suggested for epidermal cells.[32] This hypoth-

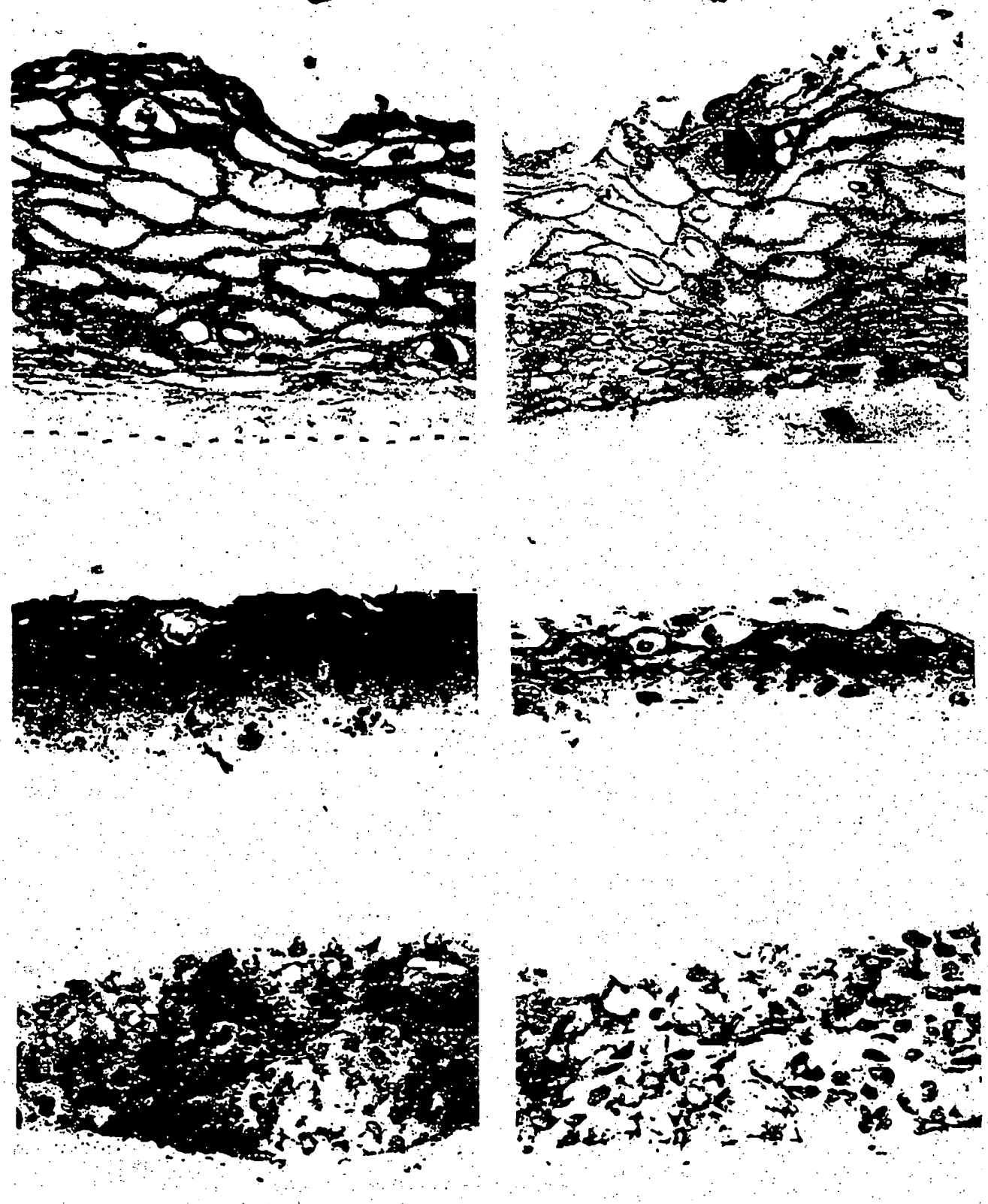

FIGURE 2. Expression of L1 and CD36 by cervical keratinocytes in organotypic tissue culture: top left and right, NCx; center left and right, W12; bottom left and right, CaSki. Top left, center left, bottom left: L1; top right, center right, bottom right: CD36. (Immunoperoxidase staining using avidin-biotin amplification and diaminobenzidine as chromogen. The base of the epithelium is indicated by a dashed line in the top left panel. Magnifications ×320.)

esis is supported by the constitutive expression of CD36 in differentiated layers of W12 and NCx cells, with no expression in monolayer culture, where the capacity for differentiation is restricted, and no expression by CaSki and SiHa cells, which display a limited differentiation program even in organotypic tissue culture. Expression by differentiated keratinocytes may be related to activation of intracellular signalling pathways or to autocrine or paracrine stimulation from cytokines released on differentiation. Either of such pathways may be modulated as part of the cytopathic effects of HPV on superficial keratinocytes in low-grade cervical lesions, although this has not been investigated in vitro.

The functional role of CD36 expression by cervical keratinocytes remains unclear. The molecule has been claimed to contribute to lymphocyte:keratinocyte adhesion in the epidermis,[12,53] but we do not believe that CD36 plays a significant role in the initial interactions required for T-cell trafficking into the ectocervix, which involves binding of lymphocytes to basal keratinocytes. Monoclonal antibodies to CD36 produced no reduction in lymphocyte binding to cervical keratinocytes in monolayer culture (data not shown), whereas antibodies to ICAM1 were able to reduce binding by 50%.[10] It remains conceivable, however, that CD36 has a role in lymphocyte retention in the superficial ectocervical epithelium, where CD36 is expressed in some cases in vivo.

CD36 on keratinocytes also may function as an immunologic accessory molecule, similar to its role on CD36+, HLA-DR+, CD11b− monocytes.[14] As well as being able to present soluble antigen, these cells are able to activate autologous T cells in the absence of added antigens or mitogens, and such an autologous mixed lymphocyte reaction is associated with the development of suppression of immune responsiveness.[54,55] The CD36+ keratinocytes in cases of NCx and in the LG-SILs did not express CD11b (data not shown) and it is conceivable that they may act in an immunoinhibitory capacity in these lesions. HLA-DR is expressed by keratinocytes in some LG-SILs (Coleman et al, unpublished observation), but we saw no CD36+, HLA-DR+ keratinocytes in the 14 cases we studied. Nevertheless, as CD36+ cells are present in LG-SILs the induction of any local immune response may induce the CD36+, HLA-DR+, CD11b− phenotype, which may then serve to suppress the reaction before any amplification phase can ensue.

Our results with biopsy specimens from cases of NCx confirm that L1, a major constituent of myelomonocytic cells, also is expressed by keratinocytes of normal mucosal squamous epithelia.[25] The preservation of staining in LG-SILs, with reduced expression in HG-SILs, is consistent with findings from studies of squamous neoplasia at other anatomic sites.[56-58] Such differential expression patterns on cervical keratinocytes in vivo are mirrored by the in vitro findings that L1 is only patchily present on the high-grade epithelium produced by CaSki and SiHa cells, but is strongly expressed on differentiated layers of NCx and W12 cells. Our observations suggest that, as for CD36, the pattern of expression of L1 by cervical keratinocytes is most likely to be related to their differentiation status rather than representing an effect of exogenous factors, with lack of expression in HG-SILs reflecting the relative lack of differentiation of the neoplastic squamous epithelial cells.

Several functions have been ascribed to the L1 protein. It shows antimicrobial activity at biologic levels in vitro[39,40] and may be active against organisms that enter the cytosol. The antiviral properties of L1 have not been investigated, but it is conceivable that the molecule may function at least partly in the defence of cells, such as keratinocytes against viruses, including HPVs. Furthermore, L1 is a member of the growing family of S-100 proteins, which appear to be involved in cell cycle progression and cell differentiation,[41] and there is evidence that it can inhibit the proliferation of a number of transformed and nontransformed cell lines, possibly by inhibition of casein kinase II.[42] The absence of immunodetectable L1 from high-grade cervical disease therefore may not only be a morphologic correlate of restricted keratinocyte differentiation, but it also may represent a demonstrable intracellular event of importance in the neoplastic progression of such lesions.

We have presented evidence that normal ectocervical keratinocytes express both the L1 and CD36 antigens, albeit at different frequencies. The expression of both molecules is maintained in LG-SILs, in which significant infiltration by host immune cells is absent,[5] but is lost in HG-SILs, which are associated with an immune cell response.[6] Our in vitro data suggest that this loss of expression is more likely to result from changes intrinsic to the keratinocyte rather than representing the effect of external factors, such as those derived from infiltrating cells. The functional implications of these observations is unclear, but the pattern of expression we have observed may reflect alterations in intracellular signalling pathways contributing to the neoplastic process, as well as changes likely to influence the interactions of cervical keratinocytes with immune cells and possibly with intracellular infectious agents.

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Human PATHOLOGY

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Chinese Medical Journal, 109(11): 854–858, 1996.

DIFFERENT SUSCEPTIBILITY OF CERVICAL KERATINOCYTES CONTAINING HUMAN PAPILLOMAVIRUS TO CELL-MEDIATED CYTOTOXICITY

Wu Rong 吴 荣, Nicholas Coleman and Margaret Stanley

Objective. To detect the factors responsible for the susceptibility of cervical keratinocytes infected with human papillomavirus (HPV) to non-specific lysis mediated by natural killer (NK) and lymphokine activated killer (LAK) cells.

Materials and Methods. Five cervical keratinocyte lines: CaSki, SiHa, HeLa (representing high grade squamous intraepithelial lesion (HSIL)), W12 (representing low grade squamous intraepithelial lesion (LSIL)) and NCx, (normal cervix) were used as target cells in the four-hour lactate dehydrogenase (LDH) release cytotoxicity assay. The effector cells were NK and LAK. The modulatory effects of interferon gamma (IFNγ) and tumor necrosis factor α (TNFα) pretreatment of keratinocytes were investigated by adding IFNγ or TNFα into the flasks of target cells 48 hours before the cytotoxicity assays. The blocking effects of anti-intercellular adhesion molecule-1 (ICAM-1) and anti-lymphocyte function-associated antigen-1 (LFA-1) monoclonal antibodies (Mabs) were also studied.

Results. All the 5 cervical keratinocytes were susceptible to LAK, but not to NK. The sensitivity varied among the cell lines. LAK had better killing effects on HSIL than on LSIL. Pretreatment of target cells with IFNγ and TNFα increased the killing mediated by LAK, but had little effect on NK activity. Anti-ICAM-1 and anti-LFA-1 Mabs inhibited LAK-mediated cytotoxicity.

Conclusions. All the HPV infected keratinocytes used in the experiments are NK-resistant and LAK-sensitive cells. IL-2, IFNγ and TNFα play some critical roles in the regulation of the susceptibility of cervical keratinocytes, especially HSIL to LAK-mediated cytotoxicity in vitro.

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Cervical carcinoma is one of the most common malignant tumors in women worldwide. Human papillomavirus (HPV) infection has been confirmed to be an important etiological agent for cervical intraepithelial lesion and invasive cancer. The local immune response within the HPV infected lesions may be related to the clinical outcome.

It has been shown that cervical keratinocytes infected with HPV16 are able to bind peripheral blood lymphocytes (PBLs) activated by IL-2.[1] The binding was increased by interferon gamma (IFNγ) pretreatment of target cells in a dose-dependent manner. It could be inhibited by anti-intercellular adhesion molecule-1 (ICAM-1) and anti-lymphocyte function-associated antigen-1 (LFA-1) monoclonal antibodies (Mabs), suggesting that ICAM-1 may be a critical adhesion molecule in the interaction.

In this study, we extendcd these observations by examining 1) the susceptibility of cervical keratinocytes to non-specific lysis mediated by natural killer (NK) and lymphokine activated killer (LAK) cells; 2) the modulatory effects of IFNγ and TNFα (tumor necrosis factor α) pretreatment of keratinocytes, and 3) the blocking effects of anti-ICAM-1 and anti-LFA-1 antibodies. Lysis was quantified by a non-isotopic method based upon the colorimetric detection of lactate dehydrogenase (LDH) released from the lysed cells in a four-hour cytotoxicity assay.

MATERIALS AND METHODS

Preparation of lymphocytes. Buffy coats from

Department of Pathology, Qingdao Medical College, Qingdao 266021 (Wu R)

Department of Pathology, University of Cambridge, UK (Coleman N and Stanley M)

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normal human volunteers were obtained from Cambridge Blood Transfusion Centre. Lymphocytes were isolated by density gradient centrifugation on Histopaque (density: 1.083). After washing twice in $RPMI_{1640}$ medium, PBLs were cultured overnight at 2×10^3/L in $RPMI_{1640}$ containing 20% Fetal Calf Serum (FCS) at 37℃ in a 5% CO_2, 95% air atmosphere. After removing adherent cells, the NK activity of these lymphocytes was assessed. Activation of LAK cells was achieved by culturing in the above-mentioned medium containing 100 units/ml of IL-2 (British Biotechnology) for four days.

Target cells. We selected 5 cervical keratinocyte lines which were well or poorly differentiated and infected with different subtypes of HPV or different copies of the virus in order to observe if there was any relationship among them.

Cervical carcinoma derived keratinocytes, CaSki (HPV16+, 300–500 copies), SiHa (HPV16+, 1 copy) and HeLa (HPV18+, 100 copies), were maintained in continuous culture in Glasgow's modification of Eagle's medium (GMEM) containing 10% FCS at 37℃ in a 5% CO_2 incubator. They were used as models of high grade squamous intraepithelial lesion (HSIL) according to the Bethesda system.[2]

W12, as a model of low grade squamous intraepithelial lesion (LSIL), is a cervical keratinocyte line which is immortalised but non-transformed by natural infection with HPV16.[3] It was derived from non-malignant cervical tissue which was diagnosed colposcopically and histologically as LSIL. It contained about 100 copies of HPV16 DNA in the episomal form. Cell culture was performed using lethal irradiated Swiss G3T3 mouse fibroblast feeder cells and GMEM medium containing epidermal growth factor (EGF) at a concentration of 10 mg/L and 10% FCS.

Normal ectocervical keratinocytes (NCx) were obtained from hysterectomy specimens, in which there was no abnormal finding morphologically (all patients had recently documented normal cervical smears). They were confirmed to be HPV-negative by PCR.

K562 cell line was derived from a patient with chronic myelogenous leukemia in blast phase. It was used as a known NK sensitive cell line. The cells were cultured in $RPMI_{1640}$/10% FCS medium.

Pretreatment of target cells. IFNγ (Genzyme) was added to the target cells at a concentration of 300 units/ml 48 hours before the cytotoxicity assays. TNFα (British Biotechnology) was used to treat the target cells in a dose of 200 units/ml 48 hours prior to the experiments.

Cytotoxicity assay. An aliquot of the desired target cell line was removed by trypsinization. After washing, the cells were resuspended in $RPMI_{1640}$ /2% bovine serum albumin (BSA) with the dilution of 10^2/L. Effector cells in defferent dilutions (2×10^2–5×10^3/L) were introduced into 96-well round-bottomed microplates in 50 μl of $RPMI_{1640}$/2% BSA. Target cells were added in a volume of 50 μl of $RPMI_{1640}$/2% BSA at a dilution of 10^2/L with or without pretreatment. After incubating at 37℃ in a 5% CO_2, 95% air incubator for 4 hours, the plates were centrifuged at a low speed for 3 minutes, after which, 50 μl of the co-incubated cell supernatant was transferred into a flat-bottomed plate. Reagents in CytoTox 96™ Non-Radioactive Cytotoxicity Assay Kit (Promega) were used according to the manufacturer's instructions. The absorbance was recorded at a wavelength of 490 nm on an Elisa reader (Dynatech MR5000). The percentage of cytotoxicity was calculated with the formula given by the manufacturer:

$$\%.\,\text{Cytotoxicity} = \frac{\text{Experimental OD} - \text{Effector spontaneous OD} - \text{Target spontaneous OD}}{\text{Target maximal OD} - \text{Target spontaneous OD}} \times 100\%$$

Blocking assay. Anti-ICAM-1 (British Biotechnology) monoclonal antibody (Mab) was added to target cells at 37℃ at the concentration of 20 mg/L 30 minutes before addition of lymphocytes. Anti-LFA-1 Mab (British Biotechnology) was added to lymphocytes at 37℃ at the concentration of 10 mg/L 30 minutes before the co-culture assay. Anti-HLA.ABC and anti-HLA.DR Mabs (Dako)

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at the concentrations of 0–40 mg / L were also used in an attempt to block the cell lysis.

RESULTS

There was some variation among the results from different donors. The representative graphs shown in Figs. 1–4 were selected from at least 3 repeated experiments.

The cytotoxicity mediated by freshly isolated PBLs on CaSki, SiHa and K562 (Fig. 1). After PBLs were isolated and cultured in vitro overnight without IL–2 pretreatment, they were used as NK cells. NK cells were effective to kill K562 cells but had little effect on CaSki and SiHa with or without IFNγ pretreatment. NK did not show cytolytic effects on W12 and NCx (data not shown).

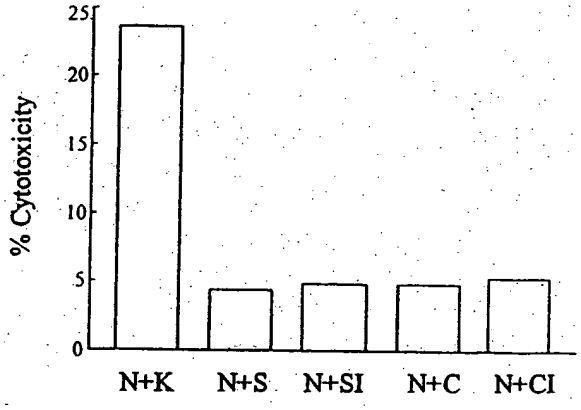

Fig. 1. The cytotoxicity mediated by NK (N) to K562 (K), SiHa (S) and CaSki (C). SI: SiHa pretreated by IFNγ (I); CI: CaSki pretreated by IFNγ (I). E:T (effector:target) = 10:1.

The cytotoxicity produced by LAK on CaSki SiHa, HeLa, W12 and NCx (Fig. 2). It was shown that LAK cells had a broader range of target cell destruction. The activity was effector:target (E:T) ratio–dependent and cell line–dependent. Satisfactory cytotoxicity was produced at an E:T ratio of 10:1 or more. The cytolytic effects on HeLa, CaSki and SiHa were better than those on W12 and NCx.

The effects of IFNγ and TNFα pretreatment of target cells on cytotoxicity (Fig. 3). IFNγ was able to upregulate the susceptibility of HSIL (SiHa, CaSki and HeLa) to LAK. Pretreatment of target cells with TNFα also improved the killing mediated by LAK.

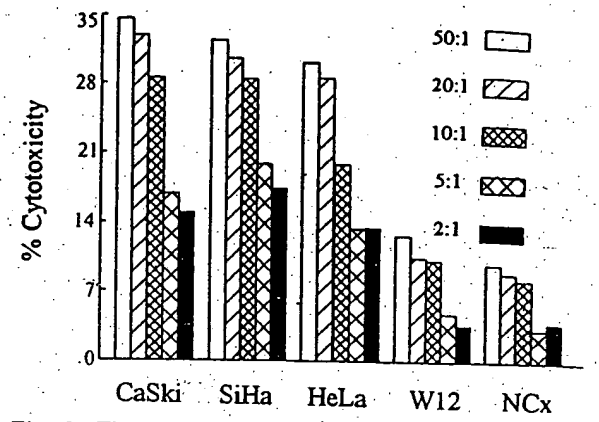

Fig. 2. The cytotoxicity produced by LAK to CaSki, SiHa, HeLa, W12 and NCx with different E:T ratios.

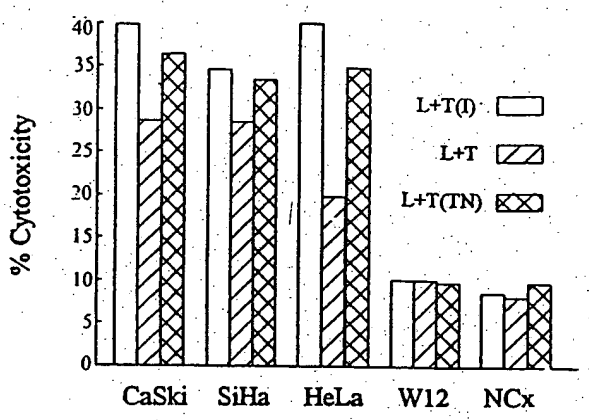

Fig. 3. The effects of IFNγ (I) and TNFα (TN) pretreatment of target cells (T) on cytotoxicity. E:T = 10:1.

The blocking effects mediated by anti–ICAM–1, anti–LFA–1, anti–HLA.ABC and anti–HLA.DR Mabs (Fig. 4). Both anti–ICAM–1 and anti–LFA–1 Mabs were able to block the cytotoxicity. Ten mg / L for anti–ICAM–1 and 5 mg / L for anti–LFA–1 Mabs were optimal for the blockage. But no similar result was found in all cases of preincubation with anti–HLA.ABC and anti–HLA.DR Mabs.

DISCUSSION

In the study of immune surveillance mechanisms in virus infection and neoplastic transformation, a growing body of evidence suggests that NK cells are involved in the defense against viral infection and in the destruction of human neoplastic cells without prior sensitization.[4] Although the killing

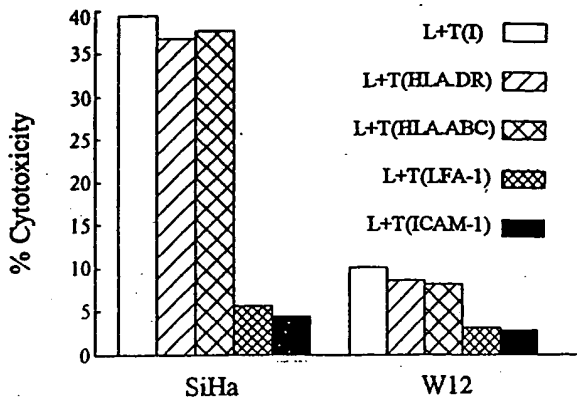

Fig. 4. The blocking effects produced by Mabs on cytotoxicity mediated by LAK to target cells. E:T = 10:1.

effect is antibody-independent and non-MHC restricted, the targets for NK are very limited. It has been reported that HPV16-immortalized cervical carcinoma cell line (QGU) is resistant to natural killing of NK cells, but sensitive to LAK cells.[5] In our experiments, freshly isolated PBLs were effective to lyse K562 but minimally responsive to CaSki and SiHa whether with or without IFNγ pretreatment of target cells, suggesting that CaSki and SiHa were NK resistant.

LAK cells are similar to NK cells in morphology but more effective than NK in mediating cytotoxicity. The target cells include both NK-sensitive and NK-resistant cells, which are not HLA-restricted and not limited to tumor cells. In our report, LAK activity against cervical keratinocytes was shown to be E:T ratio-dependent and cell line-dependent. LAK showed better killing effects on HSIL than on LSIL. It seemed that the susceptibility was correlated with neither the subtype of HPV infection nor the copies of the virus. So we think it is the poor differentiation that makes the cells more sensitive to LAK mediated killing.

It has been suggested that the interaction of LFA-1 / ICAM-1 is the principal pathway of T cell adhesion to target cells.[6] LFA-1 is expressed by all leukocytes, and is a member of the integrin family. Its counter receptor on target cells is ICAM-1. The expression of ICAM-1 on the surface of both hematopoietic and non-hematopoietic cells is selectively induced or increased within hours by cytokines such as IFNγ and TNFα. In another report, HSIL showed strong expression of ICAM-1 on full thickness of epithelium, while in LSIL, ICAM-1 expression was limited to the basal layers of epithelium.[1]

To understand the LFA-1 / ICAM-1 adhesion effect in the cytotoxicity, we incubated target with anti-ICAM-1, and LAK with anti-LFA-1 Mabs. We found that the antibodies could effectively block the killing effect in both IFNγ pretreated and untreated targets. However, no overall blocking effect in cytotoxicity has been found in cases using anti-HLA.ABC and anti-HLA.DR Mabs preincubation. The results are consistant with the findings that anti-ICAM-1 and anti-LFA-1 Mabs blocked in vitro lymphocyte:keratinocyte adhesion, while anti-HLA.ABC and anti HLA.DR Mabs had no such blocking effect.[1]

There are contradictory results in the published data about the effectiveness of IFNγ on target cell susceptibility to lysis by LAK. Some studies indicated that IFNγ treatment of certain tumor cells increased their susceptibility to LAK,[7] while others showed no change or even protection of these cells from lysis.[8] Our results demonstrated that IFNγ upregulated the targets' susceptibility to LAK, especially HSIL. We consider that the increase may be partially attributed to ICAM-1 antigen.

TNFα possesses a wide range of biologic activities,[9] one of which is the ability to mediate cytotoxicity both in vitro and in vivo. Some reports showed that most cells derived from cervical malignancies are resistant to the direct cytolytic effects of TNFα.[10] In our assays, we observed that TNFα and IFNγ showed similar effects on the cytotoxicity mediated by LAK. Perhaps it was because that TNFα and IFNγ shared some biological activities and they were positively interacted in many systems. TNFα promoted the systhesis of IFNγ and this could explain the increased sensitivity of target cells to LAK lysis.

In conclusion, our results indicated that IL-2, IFNγ and TNFα played some critical roles in the regulation of the susceptibility of cervical keratinocytes, especially HSIL to LAK-mediated cytotoxicity in vitro. HSIL cells infected with HPV showed the best susceptibility to LAK-mediated

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cytotoxicity. Whether these factors are relevant to clinical application is under investigation.

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Effect of Stapled Anastomosis in Surgery of the Esophagus–experience of 1605 Cases. Sun Yue, et al. Dept Thorac Surg General Hospital of PLA, Beijing 100853. Chin J Thorac Cardiovasc Surg 1996; (12)1:20.

Leakage at the anastomotic site in esophageal surgery remains an annoying problem to both patients and thoracic surgeons. Many papers have reported the advantages of using the stapling device in esphageal anastomosis. In order to reveal the merits of stapling anastomosis in exophageal surgery, the authors summarized the experience of stapling anastomosis in the resectional treatment for carcinoma of the esophagus and gastric cardia on 1605 cases during the period from August 1989 to February 1994. There were 1281 males and 324 females with a ratio of 3.95:1. Their age ranged from 28 to 81 years with 1184 patients (73.80%) belonging to the 50–69 age group. Carcinoma of the esophagus was present in 1044 patients and carcinoma of the cardia in 561. The anastomosis was performed in the cervical region in 35 cases and intrathoracically in 1570. Anastomotic leakage occurred in 16 patients with an overall incidence of 1% (16 / 1605). In comparison, the incidence of anastomotic leakage in the cervical anastomosis group was 14.3% (5 / 35) vs 0.7% (11 / 1570) in the intrathoracic anastomosis group. However, the incidence of intrathoracic anastomotic leakage had been as high as 1.4% (8 / 575) prior to 1986, but thereafter, the incidence came down to 0.3% (3 / 995) Anastomotic stricture occurred in 16 patients (1%). The clinical experience indicated that stapling anastomosis with a stapling device is a very effective procedure in reducing the incidence of esophageal anastomotic leakage especially in the intrathoracic anastomosis.

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In Vitro and *in Vivo* Inhibition of Human Papillomavirus Type 16 *E6* and *E7* Genes

Theresa M. C. Tan[1] and Robert C. Y. Ting

Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore

ABSTRACT

Human cervical cancers are often associated with human papillomavirus (HPV). In HPV-positive cervical cancers, the oncoproteins E6 and E7 are consistently expressed. In this study, the effects of antisense inhibition of both proteins were examined. Phosphorothioate oligonucleotides (ODNs) AE6 and AE7 complementary to regions flanking the start codons of HPV16 *E6* and *E7* genes, respectively, were synthesized. These anti-HPV ODNs inhibited the growth of cervical cell lines CaSki and SiHa, which harbor HPV16 but had little effect on cells that do not. Both ODNs also affected the ability of CaSki cells to form colonies in soft agar. In nude mice, treatment with either AE6, AE7, or a mixture of both led to substantially smaller tumors. AE7 was observed to inhibit E7 synthesis. The AE6 ODN probably exerts its effect by suppressing the expression of E6 as well as E7. Cell cultures and tumors treated with AE6 showed a decrease in E7 expression. In addition, an antisense ODN targeted at the retinoblastoma gene was able to reverse some of the inhibitory effect of AE6 on CaSki cells, indicating that AE6 inhibited E7 synthesis. This study further demonstrates that anti-HPV ODNs may be useful therapeutically.

INTRODUCTION

The antisense concept involves the use of short complementary ODNs[2] to interfere with the function of mRNAs. Such ODNs are usually between 12–20 bases in length. Hybridization of the antisense ODN with the target RNA by complementary base pairing provides high specificity and binding affinity resulting in the inhibition of expression of the protein product. The efficacy of antisense inhibition has been demonstrated for a variety of genes, particularly oncogenes (1–3).

HPVs are small DNA viruses with genomes of approximately 8 kb. Among the HPVs, a small subgroup including HPV16 and HPV18 are associated with cervical cancers (4). The transforming properties of these HPVs are encoded by the *E6* and *E7* genes (5–7). Both proteins may also play a role in the maintenance of the fully transformed phenotype.

Previous studies had shown that antisense ODNs to HPV16 and HPV18 E6 and E7 messages were useful in inhibiting cell growth and in affecting the transformed phenotype (8–10). However, the actual mechanism by which the anti-E6 and anti-E7 ODNs exert their effects is not well elucidated. We had shown previously that antisense ODNs complementary to *E6* and *E7* genes could specifically inhibit the proliferation of a cervical carcinoma cell line, CaSki (10). This study was carried out to further investigate the antisense inhibition of E6 and E7 in human carcinoma cells lines in culture, as well as to examine the effects of these ODNs on tumor growth in athymic nude mice.

MATERIALS AND METHODS

Synthesis of ODNs. Five different deoxyoligonucleotides were used in this study (Fig. 1). Two of these, AE6 and AE7, hybridize with sequences flanking the start codons of HPV16 E6 and E7 open reading frames, respectively. A sense ODN, HPVC, a randomized ODN, MA, and an ODN complementary to the retinoblastoma gene, ARb, were used as controls. PS ODNs of all five ODNs were custom made by Oligos Etc Inc. Before adding to the cell culture, the PS ODN was preincubated with the transfection reagent, N-[1-(2,3-Di-oleoyloxy)propyl]-N,N,N-trimethyl ammonium methylsulfate, DOTAP (Boehringer Mannhein, Mannhein, Germany), at 5 μg/ml for 10 min.

Cell Lines. Two established cervical epithelial tumor cell lines, CaSki and SiHa, containing HPV16 were used in this study. Two other cervical tumor lines, C-33A, lacking the HPV genome, and HeLa, containing HPV18, were used as control lines. CaSki, SiHa, and HeLa cells were grown in MEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. C-33A cells were grown in a similar medium with the addition of 0.8 mg/liter of MEM nonessential amino acids and 1 mM pyruvate.

ODN Uptake. The AE7 PS ODN was conjugated at the 5′-terminus with biotin. Cells were seeded at a density of 2×10^3 cells/well in 16-well tissue culture chamber slide vessels (Nunc, Roskilde, Denmark). After 24 h, the medium was changed, and fresh medium containing 10 μM AE7 and 5 μg/ml DOTAP was added. At 1, 4, 8, and 24 h after the addition of the ODN, the medium was removed. Cells were washed several times with PBS and fixed with 80% acetone for 10 min. The cells were then rinsed in PBS and incubated with fluorescein-conjugated strepavidin (Dako A/S, Glostrup, Denmark) for 30 min. The cells were washed again with PBS and immediately viewed by using a UV-fluorescence microscope.

Growth Assay. Cells (2×10^3) were seeded in each well of the 96-well plate and allowed to recover for 24 h before treatment with PS ODNs. Fresh medium containing 5 μg/ml DOTAP and various concentrations of PS ODNs were then introduced. Forty-eight h after the treatment, the cells were subjected to MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) assay as described previously (10). All assays were performed in triplicates.

Anchorage-independent Growth. CaSki cells were grown in 35-mm dishes until 70–80% confluence. The cells were then treated with 20 μM of PS ODNs for 4 h, resuspended in fresh medium, and 6×10^3 viable cells were seeded on a 0.5% agarose medium and allowed to grow for 3 weeks. Colonies >100 cells were then counted. All assays were performed in duplicates, and two independent experiments were carried out.

Tumor Growth in Nude Mice. Female congenitally athymic mice, (BALB/c, *nu/nu*) between the ages of 6–8 weeks were used. Five $\times 10^6$ SiHa cells were resuspended in 0.1 ml of PBS and injected s.c. into the right hind leg of each mouse. Tumors were first visible after 10 days. Once palpable tumors were established, the mice were given injections with PS ODNs at the tumor site. At the end of the experiment, the mice were killed by N_2 euthanasia. Each tumor was then excised and weighed.

Immunological Procedures. For immunoblotting, cellular lysates from cells treated with 20 μM of PS ODNs were prepared in the lysis buffer. The lysis buffer consisted of PBS (pH 7.5) containing 0.1% SDS, 1% NP-40, 2 mM EDTA, 100 μM Na_3VO_4, 0.01% phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. The lysates were clarified at 15,000 × *g* at 4°C for 10 min and stored at −20°C. Homogenate of tumor tissues from nude mice were also prepared in the lysis buffer and cleared by centrifugation as described. Protein concentrations were determined by using the Bio-Rad (Bio-Rad Laboratories) protein assay. Samples were fractionated on SDS-PAGE and transferred onto nitrocellulose membrane. A mouse mAb was used to detect E7 (Ciba-Corning Diagnostics Corp.), while two rabbit polyclonal antibodies were used to detect pRb (Oncogene Science) and actin (Sigma Chemical Co.). The bound antibodies were visualized by using enhanced chemiluminescence (ECL; Amersham).

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[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: ODN, oligonucleotide; PS ODNs, phosphorothioate analogues; HPV, human papillomavirus.

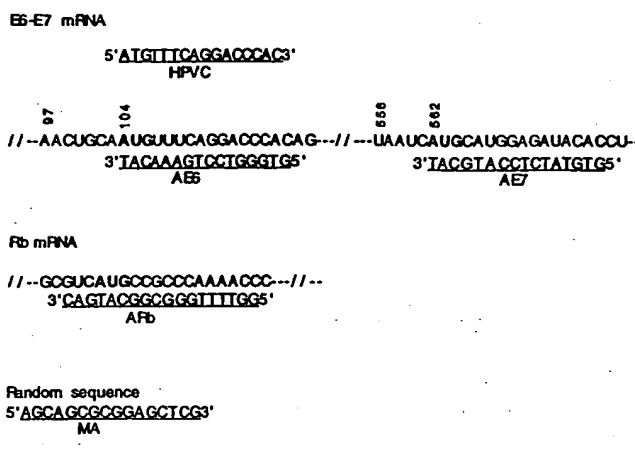

Fig. 1. Sequences of oligonucleotides used in this study and position of base pairing to the E6-E7 mRNA and Rb mRNA. HPVC correspond to the sense sequence of E6.

RESULTS

Cellular Uptake of ODNs. Uptake of the biotinylated PS ODN AE7 was monitored over a period of 24 h. Fluorescence staining could be observed in SiHa cells 1 h after incubation with the ODN and was present throughout the 24-h period (Fig. 2). A similar pattern of ODN uptake was also observed for CaSki cells (data not shown).

Growth Inhibition of CaSki and SiHa Cells. In our earlier study, it was observed that 20 μM of AE6 or AE7 could selectively inhibit the proliferation of CaSki cells (10). In this study, the effects of various concentrations and combinations of ODNs on the proliferation of CaSki and SiHa cells were examined. Cells were treated with AE6, AE7, or a mixture of both in the ratio of 1:1. Concentrations ranging from 0.1 μM to 40 μM were used.

Treatment of CaSki and SiHa cells with AE6 resulted in inhibition of cell proliferation. This inhibition ranged from 6.5 to 67.2% for CaSki cells (Fig. 3*A*) and from 11.8 to 56.1% for SiHa cells (Fig. 3*B*) when the cells were treated with concentrations varying from 5 to 40 μM. Similarly, AE7 treatment also led to inhibition of cell growth ranging from 26.0 to 60.8% for CaSki cells (Fig. 3*A*) and from 21.1 to 50.2% for SiHa cells (Fig. 3*B*) at concentrations between 1 and 40 μM. However, a combination of AE6 and AE7 was more effective, and >50% inhibition was observed at 5 μM (Table 1).

Treatment of both CaSki and SiHa cells with the control ODNs MA, HPVC, or ARb led to inhibition of <20% (Fig. 3). In addition, AE6 and AE7 had little effect on the proliferation of C-33A, which does not harbor HPV DNA, and of HeLa, which contains HPV18 (Table 2). The presence of the cationic lipid DOTAP at 5 μg/ml did not affect the proliferation of all the cells.

In another series of experiments, CaSki cells were treated for 48 h with a mixture of AE6 and ARb (2:1 ratio). The presence of ARb diminished the inhibitory effect of AE6 on the proliferation of CaSki cells (Table 3).

E7 Expression in CaSki Cells. E7 expression could be detected in CaSki cells and this expression was induced by 100 ng phorbol 12-myristate-13-acetate (TPA)/ml medium. Expression of E7 was reduced after 5 h of incubation with 20 μM AE7 (Fig. 4). The decrease in E7 expression was still evident at 24 h. However, at 24 h, the reduction in E7 protein level was less than at 5 h. A similar change in E7 expression was also observed when the cells were treated with AE6. The expression of an unrelated protein, actin, was not affected at all.

Anchorage-independent Growth. Incubation of CaSki cells for 4 h with either AE6 or AE7 did not affect the viability of the cells (data not shown). However, cells that had been pretreated with either AE6 or AE7 formed fewer colonies on agar than did cells treated with MA or cells that were not treated (Table 4).

Tumor Growth in Nude Mice. At 5×10^6 cells, both CaSki and SiHa cells formed tumors in all mice. However, the latent period for CaSki cells was much longer than for SiHa and, hence, SiHa cells were used for all subsequent experiments. Once palpable tumors were established, the animals were divided into groups and treated with either PBS or with PS ODNs. In the initial experiment, the animals were treated for 10 days with 20 μg of MA, AE6, AE7, or with PBS.

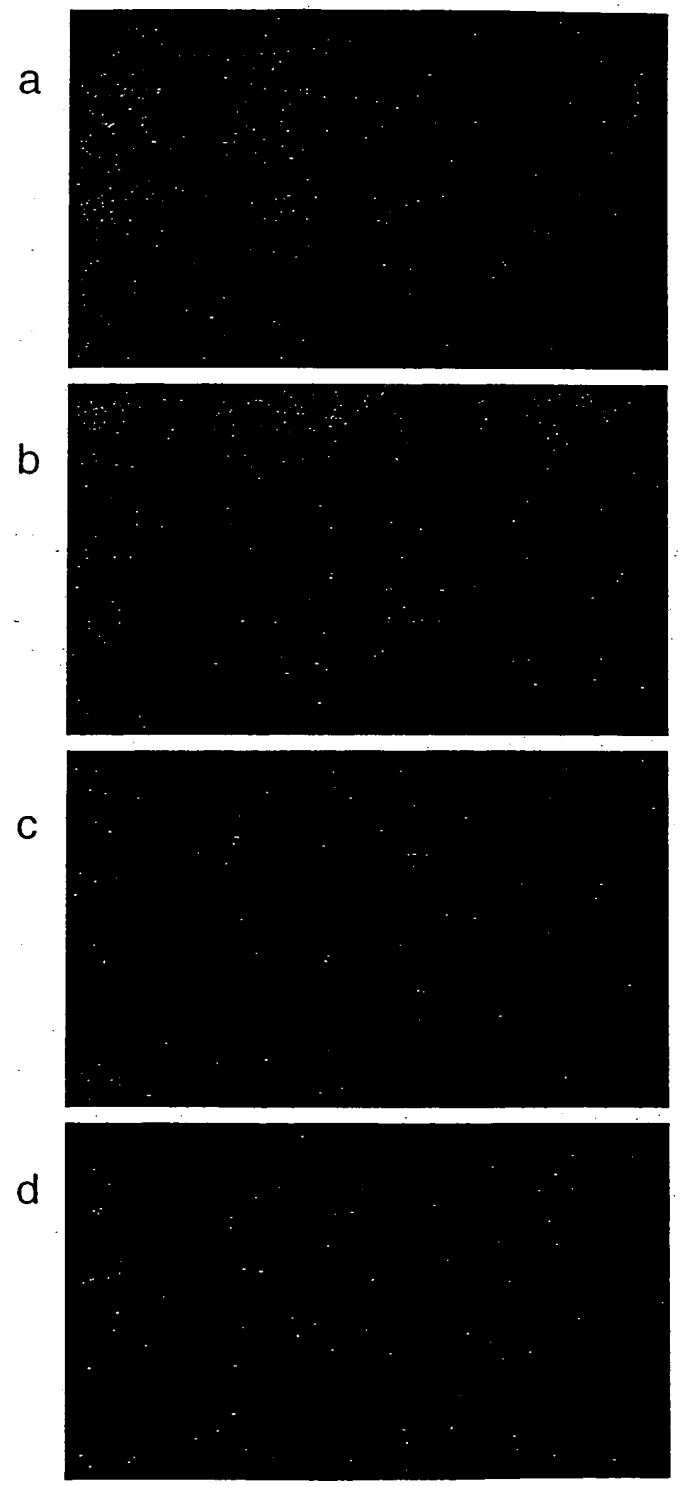

Fig. 2. Uptake of AE7 by SiHa cells at 1 (*a*), 4 (*b*), 8 (*c*), and 24 h (*d*).

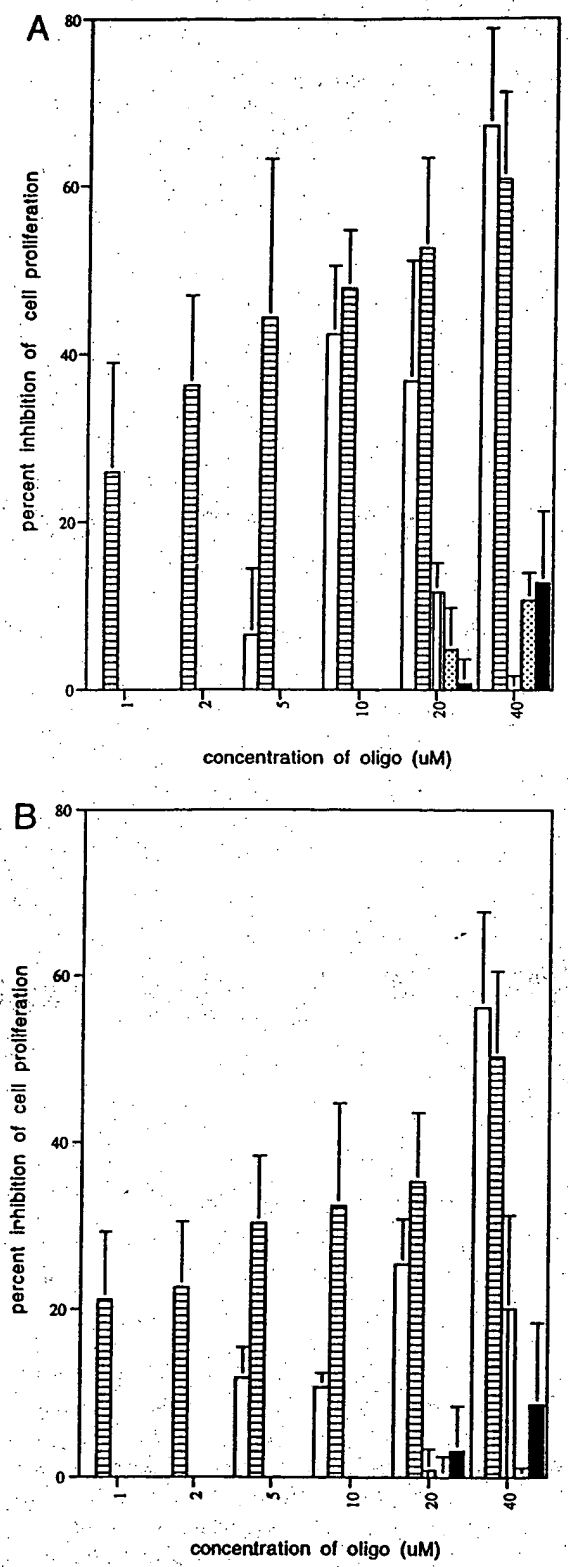

Fig. 3. Growth inhibition of CaSki cells (*A*) and SiHa cells (*B*) treated with various oligonucleotides. The cells were treated with AE6 (□), AE7 (▤), MA (▥), HPVC (▦) and ARb (■).

At the end of the treatment, animals treated with AE6 or AE7 had smaller tumors. The animals were then left untreated for 12 days before being killed. Despite the 12-day period without treatment, animals that had been previously treated with AE6 or AE7 had smaller tumors with average weight one-half that of controls (Fig. 5*A*).

In another series of experiments, all animals were treated with 30 μg of ODN daily for 15 days while the control animals were similarly treated with PBS. At the end of the treatment, animals treated with AE6 or AE7 had substantially smaller tumors (Fig. 5*B*). The average weight of these tumors was 85% lighter than those treated with PBS.

To examine if a combination of AE6 and AE7 was more effective in retarding tumor growth, the animals were treated with 10 μg of either MA, AE6, AE7, or a mixture of AE6 and AE7 in a 1:1 ratio. After 10 days of treatment, animals treated with the mixture of AE6 and AE7 had smaller tumors than those animals treated with either AE6 or AE7 (Fig. 5*C*).

Measurement of E7, pRb, and Actin in Tumors. The expressions of E7 and pRb were determined in tumors excised from the nude mice. E7 expression was reduced in tumors treated with AE6 or AE7 (Fig. 6). This decrease in E7 was accompanied by the detection of pRb. pRB was not detectable in tumors treated with PBS or MA. The expression of an unrelated protein, actin, was not affected by any of the ODNs.

DISCUSSION

In this study, we report the results of PS ODN treatments of cervical cancer cells in culture and in tumors in nude mice. The results clearly demonstrate the effectiveness of anti-HPV16 ODNs, particularly for retarding tumor growth in nude mice. Before examining the effects of the anti-HPV ODNs in nude mice, we first examined the effects on cell cultures.

In cell cultures, the uptake of the PS ODN was rapid and efficient in the presence of the cationic lipid, DOTAP. The enhancement of ODN uptake by cationic lipids has been described for a variety of cell lines (11), including CaSki (12). In this study, DOTAP was used at 5 μg/ml, and this concentration was not toxic to all the cell lines used. The ODN remained associated with the cells for up to 24 h.

Both anti-HPV ODNs, AE6 and AE7, were able to inhibit the

Table 1 *Effects of a mixture of AE6 and AE7 (1:1) on the proliferation of CaSki and SiHa cells*

Oligonucleotide concentration	% of inhibition of CaSki cell proliferation ± SD	% of inhibition of SiHa cell proliferation ± SD
0.1 μM	32.9 ± 9.1	37.5 ± 5.5
0.5 μM	40.6 ± 8.3	37.3 ± 6.4
5.0 μM	58.7 ± 11.7	50.8 ± 14.0

Table 2 *Effects of antisense oligonucleotides on the proliferation of Hela and C-33A cells*

Oligonucleotide (concentration)	% of inhibition of HeLa cell proliferation ± SD	% of inhibition of C-33A cell proliferation ± SD
AE6 (20 μM)	6.7 ± 7.1	6.6 ± 3.0
AE6 (40 μM)	13.8 ± 7.6	23.7 ± 13.3
AE7 (20 μM)	19.8 ± 8.4	0.7 ± 4.9
AE7 (40 μM)	18.8 ± 8.1	18.2 ± 3.5
MA (20 μM)	10.1 ± 8.4	ND[a]
MA (40 μM)	17.3 ± 8.1	ND
HPVC (20 μM)	16.4 ± 6.8	ND
HPVC (40 μM)	22.3 ± 7.0	ND

[a] ND, not determined.

Table 3 *Effects of antisense oligonucleotides ARb:AE6 (1:2) on the proliferation of CaSki cells*

Oligonucleotide concentration	% of inhibition of cell proliferation ± SD
30 μM	19.6 ± 2.9
40 μM	20.2 ± 8.6

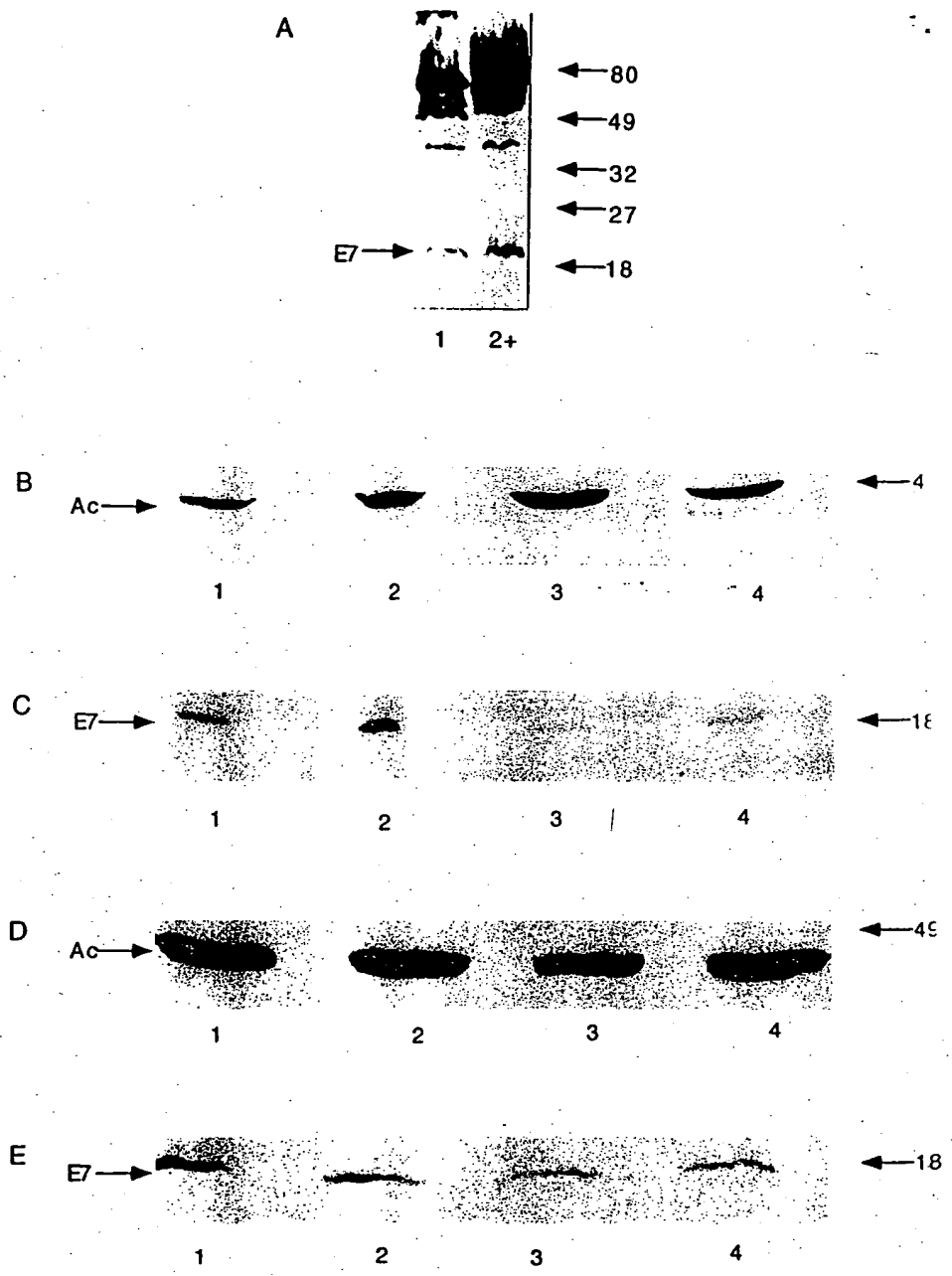

Fig. 4. Western blot analysis of E7 and actin expression in CaSki cells. *A*, detection of E7 in the absence (*Lane 1*) and presence (*Lane 2*) of 100 ng/ml TPA. *B*, actin, and *C*, E7 expression after incubation with ODNs for 5 h. *D*, actin, and *E*, E7 expression after incubation with ODNs for 24 h. *Lane 1*, lysate of untreated CaSki cells; *Lane 2*, lysate of cells treated with a control ODN, MA; *Lane 3*, lysate of cells treated with AE7; and *Lane 4*, lysate of cells treated with AE6. The positions of *M*, markers are indicated on the *right* and the position of actin (*Ac*) and E7 are indicated on the *left*.

proliferation of CaSki and SiHa cells. Although Storey *et al.* (13) had reported nonspecific inhibition with similar ODNs, this study and that of Wong *et al.* (9) proved otherwise. Control ODNs did not significantly affect the growth of CaSki and SiHa cells, even at the highest concentration of 40 μM. In addition, AE6 and AE7 also decreased the ability of CaSki cells to grow on soft agar. The effects of AE6 and AE7 were specific to cells harboring HPV16, and there was little effect on the cervical line, C-33A, which has no HPV DNA, and HeLa, which contains HPV18.

In the nude mice, the anti-HPV ODNs specifically inhibited tumor growth and the control ODNs had little effect. Tumor growth was repressed by AE6 and AE7 throughout the entire period of treatment. At 12-days posttreatment, AE6-treated and AE7-treated animals still had substantially smaller tumors compared to the controls. The effect of AE6 and AE7 is probably due to a reversal of the transformed phenotype brought about by the inhibition of E6 and E7. Expression of *E6* and *E7* genes has been linked directly to the proliferative capacity of cervical cancer cells (14–16) and is also required for the maintenance of the transformed phenotype (6, 17).

Table 4 *Effect of antisense oligonucleotides on colony formation in soft agar*

Treatment	No. of colonies
Untreated	33.5
MA (20 μM)	35.5
AE6 (20 μM)	11.0
AE7 (20 μM)	9.5

The effects of AE7 on cell and tumor growth are associated with the suppression of E7 expression. In CaSki cells, the half-life of E7 is only 55 min (18). As such, it was possible to detect a decrease in E7 expression after only 5 h of incubation with AE7. However, the suppression of E7 expression was less after 24 h, and this led to only 50–60% inhibition of CaSki and SiHa cells after 48 h of treatment. E7 expression was also inhibited in tumors treated with AE7. This inhibition is specific to E7, and actin expression was not affected at all

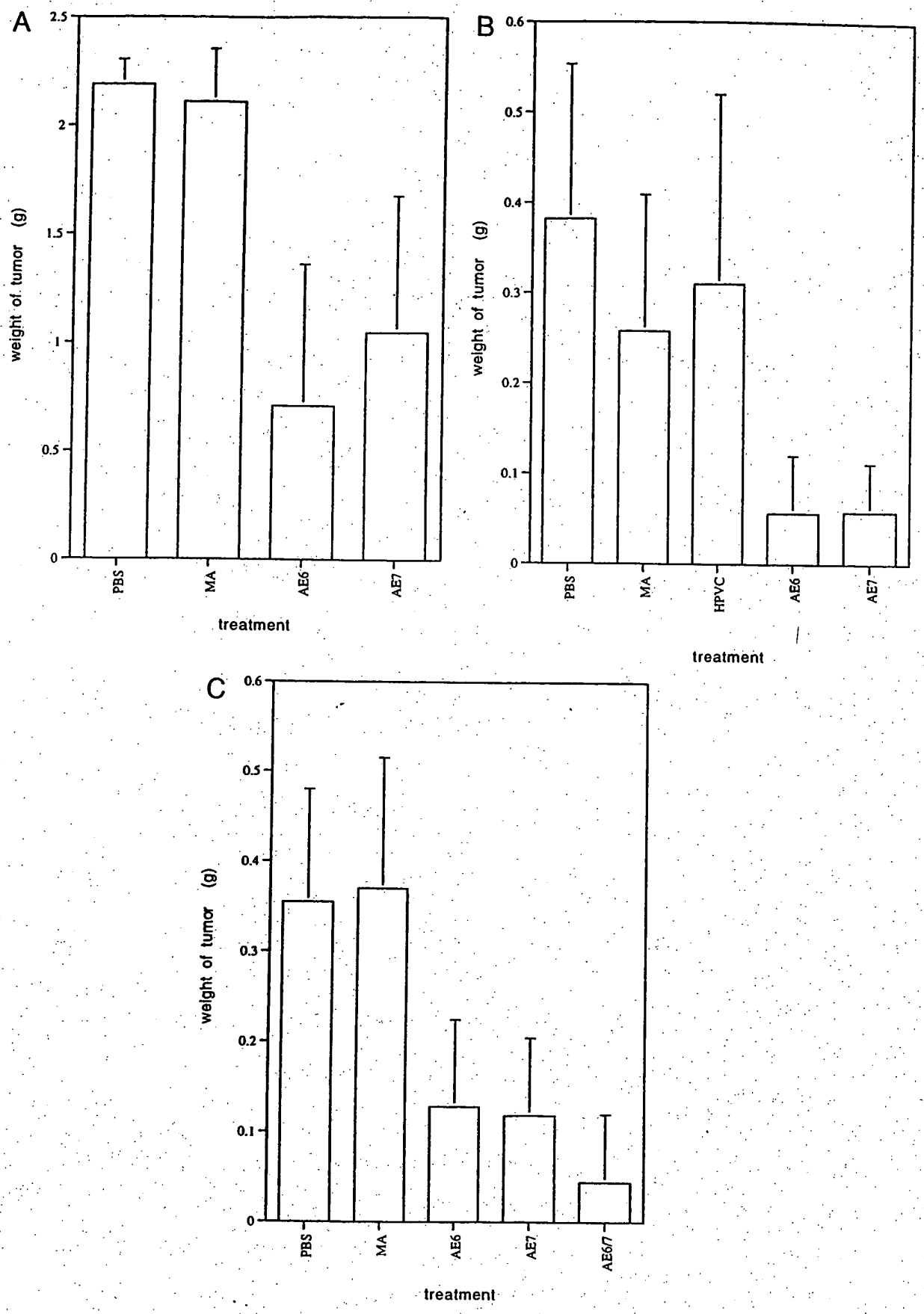

Fig. 5. Mean weight of tumor (± SD) in nude mice after s.c. injection of SiHa cells. *A*, mean weight of tumors 12 days after treatment with PBS (n = 3 mice), 20 μg of MA (n = 3 mice), 20 μg AE6 (n = 5 mice), and 20 μg AE7 (n = 5 mice). *B*, mean weight of tumors at the end of 15 days of treatment with 30 μg of oligonucleotides. Animals were treated with PBS (n = 6), MA (n = 6), HPVC (n = 6), AE6 (n = 9), or AE7 (n = 9). *C*, mean weight of tumors at the end of 10 days of treatment with 10 μg of oligonucleotides. Animals were treated with PBS (n = 4), MA (n = 7), AE6 (n = 8), AE7 (n = 5), and AE6/AE7 (n = 8).

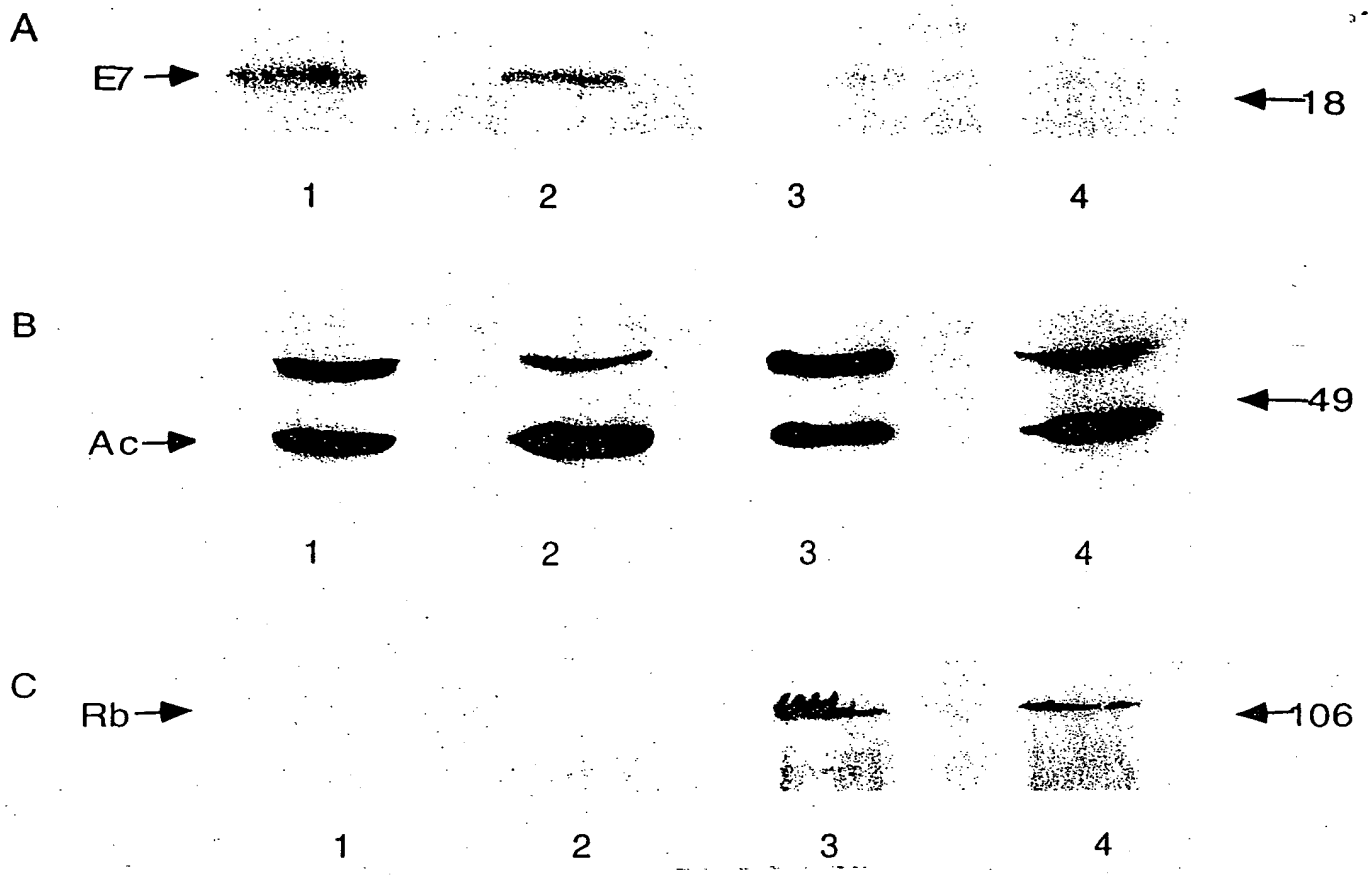

Fig. 6. Western blot analysis for E7 (*A*), actin (*B*), and pRb (*C*) proteins in tumors treated with PBS (*Lane 1*), MA (*Lane 2*), AE6 (*Lane 3*), and AE7 (*Lane 4*). The positions of *M*, markers are indicated on the *right* and the position of E7, actin (Ac), and pRB are indicated on the *left*.

The AE6 ODN probably exerts its effect through the suppression of E6 as well as the expression of E7. In cervical cancers and cervical cell lines containing HPV, mRNAs encoding E6 and E7 proteins are transcribed from the same promoter in the form of a bicistronic transcript (19, 20). *In vivo*, the bicistronic transcript is also spliced to produce two shorter transcripts E6*I-E7 and E6*II-E7 (21). It has been proposed that it is a function of these spliced transcripts to stimulate E7 translation (19, 20). Because all three transcripts have the same 5′ ODN sequence, one would assume that AE6 could affect all three because regions at the 5′ end of the mRNA are most sensitive to antisense inhibition of translation (22–24).

Our data does indeed indicate that AE6 affects all three transcripts, thereby, inhibiting E7 translation. *In vitro*, inhibition of the full-length bicistronic E6-E7 transcript by AE6 results in the suppression of both E6 and E7 expression. This was achieved by the specific hybridization of AE6 with the E6 sequence and not through nonspecific interaction of AE6 with the E7 sequence (10). E7 is known to bind the product of the retinoblastoma susceptibility gene, *pRb* (25), thereby, preventing pRb from regulating cell growth. Inhibition of E7 will, thus, allow pRb to perform its function of regulating cell growth. For CaSki cells, the inhibition of cell proliferation by AE6 can be reversed by inhibiting pRb expression by using the ODN ARb, which has been shown to be effective in inhibiting the synthesis of pRb in human embryonic lung fibroblasts (26). This reversal suggests that part of the effects of AE6 is achieved via the inhibition of E7 expression. In addition, the expression of E7 in the tumors in nude mice probably caused the Rb protein to be unstable and, hence, Rb could only be detected in tumors treated with AE6 or AE7 because both ODNs suppress E7 expression. The reduction of E7 expression in tumors and cells treated with AE6 indicates that AE6 probably also inhibits E7 expression from the two shorter transcripts, E6*I-E7 and E6*II-E7, because *in vivo*, these two transcripts account for >80% of the major E6-E7 transcripts (21).

Antisense inhibition of E6 and E7 has been shown to be useful in inhibiting the growth of cervical cells harboring HPV16 or HPV18 DNA (8–10). This study also shows that anti-E6, as well as anti-E7 ODNs, are useful in impeding tumor growth in nude mice. A combination of AE6 and AE7 was more effective than each of the ODNs individually. These observations, together with the specificity of the antisense inhibition, augurs well for the therapeutic potential of antisense ODNs in HPV-related cervical cancers.

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In Vitro Antigene Therapy Targeting HPV-16 E6 and E7 in Cervical Carcinoma

MARILU MADRIGAL, M.D.,[1] MIKE F. JANICEK, M.D.,[2] BERND-UWE SEVIN, M.D., PH.D., JAMES PERRAS, PH.D., RICARDO ESTAPE, M.D., MANUEL PEÑALVER, M.D., AND HERVY E. AVERETTE, M.D.

Division of Gynecologic Oncology, Department of Gynecology and Obstetrics, University of Miami School of Medicine, 1475 NW 12th Avenue (D-52), Miami, Florida 33136

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Human papillomavirus (HPV) infection is believed to play a central role in cervical carcinogenesis. Specifically, two viral oncoproteins, E6 and E7, possess transforming ability and have been shown to interact with the cellular tumor suppressors p53 and p105, the retinoblastoma (Rb) gene product. To test the hypothesis that E6 and E7 play an active role in the maintenance of the malignant phenotype and may be ideal targets for antigene therapy, we tested the antiproliferative effects of phosphorothioate oligodeoxynucleotides (oligos) targeting HPV-16 E6 and E7 in cervical cancer cell lines and primary tumor explants.The ATP cell viability assay was used to measure growth effects of 27-mer antisense oligos targeting the ATG translational start region of HPV-16 E6 and E7 sequences in HPV-16-positive cell lines SiHa and CaSki and four advanced, primary cervical tumor explants. A random oligo sequence, an HPV-18-positive and HPV-negative cell line, one histologically confirmed endometrial and two ovarian tumors were used as negative controls. HPV type was confirmed by hybrid capture techniques. Cell lines and sterile (staging laparotomy) tumor cells were plated at 5000 cells/0.1 ml and 100,000 cells/0.5 ml in 96-well plates or soft agar, respectively, and incubated at 37°C with a single treatment of oligos at 0–16 μM. E6/E7 combinations at a fixed ratio of 1:1 were used at 0–8 μM for each oligo. Cellular ATP was measured by luciferin/luciferase fluorescence on Day 6. HPV-16 E6 and E7 oligos showed antiproliferative effects in all HPV-16-positive cell lines and primary tumor explants (IC_{50}s 6.9–9.5 μM for cell lines, 9.1–12.1 μM primary cervical tumors), while the HPV-negative C33-A cell line and HPV-18-positive cell line HeLa were relatively insensitive to the HPV-16 oligos (IC_{50}s > 30 μM extrapolated). The endometrial and two ovarian primary tumors were also insensitive to the HPV E6 and E7 oligos (IC_{50}s > 25 μM extrapolated). Random oligos had little effect on cell growth at concentrations up to 16 μM (< 25% inhibition), except in CaSki (@50% inhibition at 16 μM). Combinations of E6 and E7 demonstrated mixed synergistic and antagonistic effects as determined by combination indices (CI) derived from median effect parameters. In the HPV-16-positive primary cervical tumors and the cell line SiHa, E6/E7 combinations were synergistic at low doses (<25% growth inhibitory dose range) and antagonistic at doses above this. For the HPV-16-positive cell line CaSki, however, E6/E7 combinations were antagonistic at all dose ranges. Phosphorothioate oligos directed against the viral oncogenes E6 and E7 were shown to have antiproliferative effects specific to HPV-containing cancer cells. These specific antiproliferative effects suggest that HPV-16 E6 and E7 sequences play an active role in the malignant growth properties of cervical cancer cells and may be ideal targets for antigene therapy.

INTRODUCTION

Advances in the understanding of the role of HPV (human papillomavirus) in cervical cancer have produced strong evidence confirming the importance of the viral–host cell interactions in the etiology of cervical carcinoma. While a variety of other factors play a role in cervical carcinogenesis, 90% of squamous cervical carcinomas contain HPV genes that are believed to function as oncogenes [1]. If these viral oncogenes are actively involved in the maintenance of the malignant phenotype, then they are ideal targets for directed antigene therapy. Targeting genetic alterations unique to cancer cells may be clinically advantageous by selective inhibition of malignant cells while sparing normal cells. If, however, HPV initiates permanent alterations which subsequently lead to the development of malignancy, then targeting viral factors should produce little benefit. Expression of HPV oncogenes has been shown to be required for the establishment *and maintenance* of the malignant phenotype in human keratinocyte cell lines [2], but the fundamental question of the *active* role of HPV in human cervical cancers remains unclear.

We sought to directly test the hypothesis that HPV oncogenes play an active role in the maintenance of malignant

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[1] To whom correspondence and reprint requests should be addressed.

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growth properties. By targeting the expression of HPV-16 E6 and E7 genes with phosphorothioate oligodeoxynucleotides (oligos) in cervical cancer cells, growth effects were examined with the ATP cell viability assay. We have utilized both cell lines and primary cervical tumor explants to test this hypothesis.

Storey *et al.* [3] demonstrated that phosphorothioate oligodeoxynucleotides complementary to various E6 and E7 target sequences have specific and nonspecific inhibitory effects using micromolar concentrations of oligos. Interestingly, however, the rate of synthesis of E6 and E7 proteins and the steady-state levels of E7 mRNA were shown to remained largely unchanged. Steele *et al.* [4] showed that oligos targeting the start codons of E6 and E7 in HPV-18 containing cell lines produced specific antiproliferative effects. Tin and Tang published two articles relating to anti-E6 and E7 oligos in cervical cancer cell lines and in nude mice using bicistronic mRNA and phosphorothioate oligonucleotides, both studies demonstrating antiproliferative effects against the CaSki cell line [5, 6].

The rationale for targeting E6 and E7 stems from the evidence that these viral oncogenes interact directly with the cellular tumor suppressors p53 and p105/Rb (the retinoblastoma gene product) [7]. E6 has been shown to cause ubiquitin-mediated proteolysis of p53, while E7 binds to and inactivates Rb [7–9]. Previous studies have shown that the most effective oligos against E6 and E7 target the translational start sequences [3, 4]. Though translation inhibition and activation of cellular RNase H's are thought to be key mechanisms, the precise action of these antisense oligos has not been determined.

We examined the effects of phosphorothioate-modified oligodeoxynucleotides which are complimentary to viral mRNA (antisense oligos) specifically targeting the E6 and E7 ATG regions of HPV-16. Fresh cervical tumor explants were confirmed to contain HPV 16 as well as cervical epithelial tumor cell lines either containing (SiHa and CaSki) or lacking (C33-A and HeLa) HPV 16. C33-A contains no HPV DNA, while HeLa contains HPV-18 DNA [10–12]. In this study we have demonstrated that the antisense oligos targeting the translation initiation sites of either E6 or E7 inhibited proliferation of HPV 16-containing cells when compared to cells lacking HPV 16.

MATERIALS AND METHODS

Cell lines. SiHa, C33-A, HeLa, and CaSki were obtained from the American Type Culture Collection. Cell lines were maintained in Eagle's modified essential medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 g/ml amphotericin B in incubators at 37°C, 95% humidity, and 5% CO_2. Confluent cell monolayers were used for subculturing and to prepare cells for oligo treatment by detaching them with 0.25% trypsin/0.02% EDTA, manual counting with a hemacytometer, and dilution with media at 37°C.

Oligos. Phosphorothioate oligodeoxyribonucleotides 27 bases in length (M_r ~8700) were synthesized by an automated DNA synthesizer (Applied Biosystems 394 DNA/RNA Synthesizer) in 1 m*M* batches to 60–70% purity. Phosphorothioate oligodeoxyribonucleotides were synthesized with all phosphodiester positions modified. Oligo preparations were ethanol precipitated under sterile conditions, the pellets were resuspended in sterile water, and concentrations were determined by OD at 260 nm, with OD 260:280 nm ratios ranging between 1.5 and 2.0, consistent with single-strand oligos. Stock solutions of oligos were then prepared by dilution with the same medium used for cell incubations.

The antisense oligos for sequences and targets for E6 and E7, along with the scrambled control sequence (random sequence), are shown in Table 1 [13, 14]. A control sequence was generated from the corresponding antisense sequence of E6 using random number tables.

Treatment of cell lines. Cells were treated in 96-well microtiter plates by first diluting oligos in sterile media and then adding the counted cell suspensions to 5000 cells/well. Final oligo concentrations were 16, 8, 4, 2, and 1 μM in a final volume of 100 μl/well. For experiments using a combination of oligos, fixed 1:1 ratios of the two oligos were used at concentrations of 8, 4, 2, 1, and 0.5 μM each. All oligo concentrations were tested in triplicate for each experiment, and each experiment was repeated a minimum of three times. Incubations were carried out for 6 days without replacement of media or oligos.

Preparation of cervical tumor cells. Fresh cervical tumor specimens were obtained intraoperatively and transported to our laboratory for processing. The four primary cervical tumors were surgical IIB, two IIIB's, and two recurrences. All involved squamous histology, and tissues were obtained from gross nodal disease or parametrial breakthrough. No tissue from the vagina/cervix itself was obtained due to the possibility of bacterial contamination. A 1- to 2-cm piece of the fresh tumor tissue was set aside prior to processing the tumor cells and tested for HPV type using hybrid capture assays specific for HPV-16 (Hybrid Capture System, HPV 16 Probe Pack)[15]. Tumors not containing HPV-16 subtypes were excluded from oligo testing.

Briefly, the fresh tumor tissue was submersed for 20 min in Hanks' balanced salt solution (HBSS) containing penicillin and streptomycin. Connective tissue and fat are then grossly dissected to minimize nonmalignant cell content. The remaining tumor tissue is then finely minced and incubated overnight in HBSS containing 5% fetal bovine serum (FBS), 1× penicillin and streptomycin, 2.25 PUK/ml pronase, 0.2 mg/ml collagenase, and 1400 U/ml DNase. Disaggregated tissue fragments and cells are then washed twice in HBSS with 5% FBS and suspended in enriched Cannaught Medical

TABLE 1
The Phosphorothioate Oligodeoxyribonucleotide Sequences Used in This Study

Oligo	Sequence (1 3 5 7 9 11 13 15 17 19 21 23 25 27)	G*C
		(%)
E6 antisense	5′ A C A T T G C A G T T C T C T T T T G G T G C A T A A 3′	37
E7 antisense	5′ T G T A T C T C C A T G C A T G A T T A C A G C T G G 3′	44
Random	5′ G T C G A C T A A T C C G T T T T G A G A T A C T T T 3′	37
	* * * * * * * * * * * * * *	% Mismatch
HPV 16 E6 consensus	5′ T T A T G C A C C A A A A G A G A A C T G C A A T G T 3′	67%
HPV 18 E6 consensus	5′ C T A T G G C G C G C T T T G A G G A T C C A A C A C 3′	
	* * * * * * * * * * * * * *	
HPV 16 E7 consensus	5′ C C A G C T G T A A T C A T G C A T G G A G A T A C A 3′	52%
HPV 18 E7 concensus	5′ A T A A T A T T A A G T A T G C A T G G A C C T A A G 3′	

Note. The anti-E6 sequence targets the E6 and E7 ATG regions of HPV 16 [18]. The sequence complimentary to the AUG translation start condon of the E6 transcript is underlined in bold. Asterisk denotes mismatch.

Research Laboratory (CMRL) media. Large tissue fragments and cell aggregates are allowed to settle for 2 min. followed by aspiration of the upper media layer, which contains both single cells and small cell clusters. Approximate viable cell counts are obtained using trypan blue dye exclusion. Cells were diluted to 200,000 viable cells/ml and aliquoted at 100,000 cells/500 μl in 24-well plates containing an agar underlayer to minimize nonmalignant cell growth. Use of agar underlayers has been shown to effectively abolish the growth of nonmalignant cells such as fibroblasts [16].

Treatment of primary tumor cells. Serial dilutions of 16, 8, 4, 2, and 1 μM of each oligo (E6 and E7), as well as Day 0 and 6 untreated controls, were plated out in triplicate on 24-well plates containing 100,000 cell/well with agar underlayers (total volume = 0.5 ml). Combinations of oligos at a 1:1 ratio were plated at concentrations of 8, 4, 2, 1, and 0.5 μM for each oligo. Incubations were carried out at 37°C for 6 days with no media changes or additional treatments.

ATP cell viability assay. On Day 6 of incubation, intracellular ATP was extracted from the cells in each well and quantitated by the ATP cell viability assay as previously described [17]. Cells were lysed by 1 vol of 4% trichloroacetic acid, and a 50-μl aliquot was transferred to a clear plastic tube and neutralized by 2 vol of 0.1 M Tris buffer, pH 9.2. A 20-μl aliquot of this was transferred to another clear plastic tube for luminometer reading. The luminometer (Packard Picolyte) injects 50 μl of Picozyme luciferin–luciferase complex into the tube and ATP-driven fluorescence is measured for 20 sec. Standard ATP solutions are diluted and assayed with each preparation of luciferin–luciferinase complex to establish a standard ATP curve confirming linearity over the experimental range of fluorescence detection (10^{-13} to 10^{-10} mole ATP/20 μl aliquot). Fluorescent units were normalized to untreated controls to determine percentage of control ATP.

Data analysis. IC_{50} was defined as the oligo concentration required for 50% growth reduction as compared to untreated controls and was calculated from the median effect analysis by the methods of Chou and Talalay [18]. This calculation is derived from the x intercept determined by linear regression analysis of the log (f_a/f_u) vs log(C) plot, where f_a is the fraction affected, f_u is the fraction unaffected $= 1 - f_a$, and C is the concentration. Correlation coefficients for individual ATP experiments averaged 0.957 ± 0.005 (SE), range 0.860–0.998. Since experiments from fresh primary tumors could not be repeated separately, primary tumor data were pooled for statistical purposes (see Table 2). All cell line experiments were repeated separately a minimum of three times.

Synergistic vs antagonistic effects were quantitated by combination indexes as calculated by the methods of Chou and Talalay [18], where at a given $f_a = x$, CI $= [(Dx)_1/(Dx)_1] + [(D)_2/(Dx)_2]$, with $D_{1,2}$ = experimental drug concentrations, and $(Dx)_{1,2}$ = calculated drug concentrations derived from the median effect parameters of the individual agents 1 and 2 at $f_{a=x}$. CI's less than, equal to, or greater than 1 represent synergism (supraadditive), summation (additive), or antagonism (subadditive) effects, respectively.

Statistics. Differences between IC_{50}'s of oligo–cell treatment groups were calculated by standard two-tailed t tests using Statview 4.1 on a Macintosh platform.

RESULTS

Table 2 shows the IC_{50}s ± standard errors for the cell lines and primary tumors for each of the oligos or oligo combinations tested. The IC_{50}s for the HPV-16-positive cell lines SiHa and CaSki were about fivefold lower than the corresponding IC_{50}s for the cell lines HeLa and C33-A lacking HPV-16, indicating that the inhibitory effects were spe-

TABLE 2

IC_{50} ± Standard Errors (μM) Values for Single and Combination Oligo Treatments in the Four Cell Lines Tested and the Cervical Tumors

	IC_{50} ± SE							
	E6	SE	E7	SE	E6 + E7	SE	Random	SE
Cell lines								
HPV 16(+)								
SiHa	8.72	1.12	6.70	0.49	8.38	0.96	27.94	4.28
CaSki	9.07	0.70	9.48	1.62	9.93	0.78	16.10	11.24
Ave	8.90	0.18	8.09	1.39	9.16	0.77	22.02	5.92
HPV 16 (−)								
HeLa	42.50	22.04	30.20	16.50	14.93	2.60	40.06	22.09
C33-A	67.95	22.25	128.35	87.94	71.49	22.96	130.43	49.66
Ave	55.23	12.73	79.28	49.08	43.21	28.28	85.25	45.19
Primary tumors								
HPV 16(+)								
Cx 1148	8.83		5.49		9.86		61.53	
Cx 1055	12.51		7.10		11.38		240.64	
Cx 1058	19.92		13.65		8.75		279.72	
Cx 1020	6.97		9.98		13.31		249.17	
Ave	12.06	2.86	9.05	1.79	10.82	0.99	207.76	49.46
HPV 16(−)								
Endometrial	>16		>16		36.49		>16	
Ovarian 1	26.39		26.86		458.71		62.04	
Ovarian 2	23.47		111.68		147.94		439.67	
Ave	>16		>16		92.21		>16	

Note. >16 = no dose response. IC_{50} could not be calculated from median effect parameters. The extrapolated IC_{50} values in all control (randomized oligo) treated cell lines fell beyond the experimental range of concentrations used (16 μM). IC_{50} values could not be calculated for the endometrial cell line used as control.

cific to HPV-16 homologous sequences. Random oligos produced minimal inhibition in all cell lines and primary tumors (<25% at the highest dose of 16 μM), except in CaSki, where 50% inhibition occurred at 16 μM. Calculated IC_{50}s for random oligos were significantly higher ($P < 0.05$, t test) than the E6 or E7 oligos in HPV-16-positive cells, except in CaSki ($0.06 < P < 0.18$, t test).

Anti-E6 oligos were significantly more effective than E7 in SiHa ($P = 0.04$, t test), but not in CaSki. In primary tumors, E7 oligos were generally more effective than E6, but this trend did not reach statistical significance (see Table 2). The average IC_{50}s of each oligo treatment group tended to be higher for primary tumors than cell lines. Direct comparisons between cell lines and primary tumor explants, however, are not useful given the different cell preparation and incubation conditions (see Materials and Methods).

Figure 1 shows the dose–response plots for each of the oligo treatments. As seen in Fig. 1a, the dose responses for E6 oligos targeting the HPV-16 ATG sequences cluster into two groups. The cell lines lacking HPV (C33-A) or containing HPV-18 DNA (HeLa) have flat dose responses similar to those of the random oligo controls (Fig. 1d), indicating that the absence of specific target sequences renders cell lines relatively resistant to the effects of these oligos. The HPV-16-containing cell lines SiHa and CaSki, on the other hand, demonstrate a steady dose response with >50% inhibition beginning around 8 μM oligos.

The dose–response plots for E7 demonstrate a similar clustering of curves (Fig. 1b). While the HPV-16-negative cell lines remained above 50% inhibition with relatively flat dose responses, the HPV-16-containing cell lines demonstrated steeper dose responses and >50% inhibition in the higher 8–16 μM dose range. The combination of E6 and E7, as expected, also produced two types of dose–response curves, although the separation of curves was less pronounced (Fig. 1c). The random oligos produced almost flat dose responses for all cell lines except CaSki.

To further extend our findings with cell line responses to HPV-16 oligos, four HPV-16-positive primary cervical carcinomas were treated with the same panel of HPV-16 oligos. One endometrial and two ovarian carcinomas were grown and treated in an identical fashion to serve as HPV-16-negative controls. Figure 2 again shows grouping of dose responses among the HPV-16 tumors and negative controls. While the HPV-16-containing primary tumors experienced >50% inhibition at doses of 16 μM, the non-cervical tumor controls and random oligo-treated cervical primaries demonstrated minimal growth inhibition at similar concentrations.

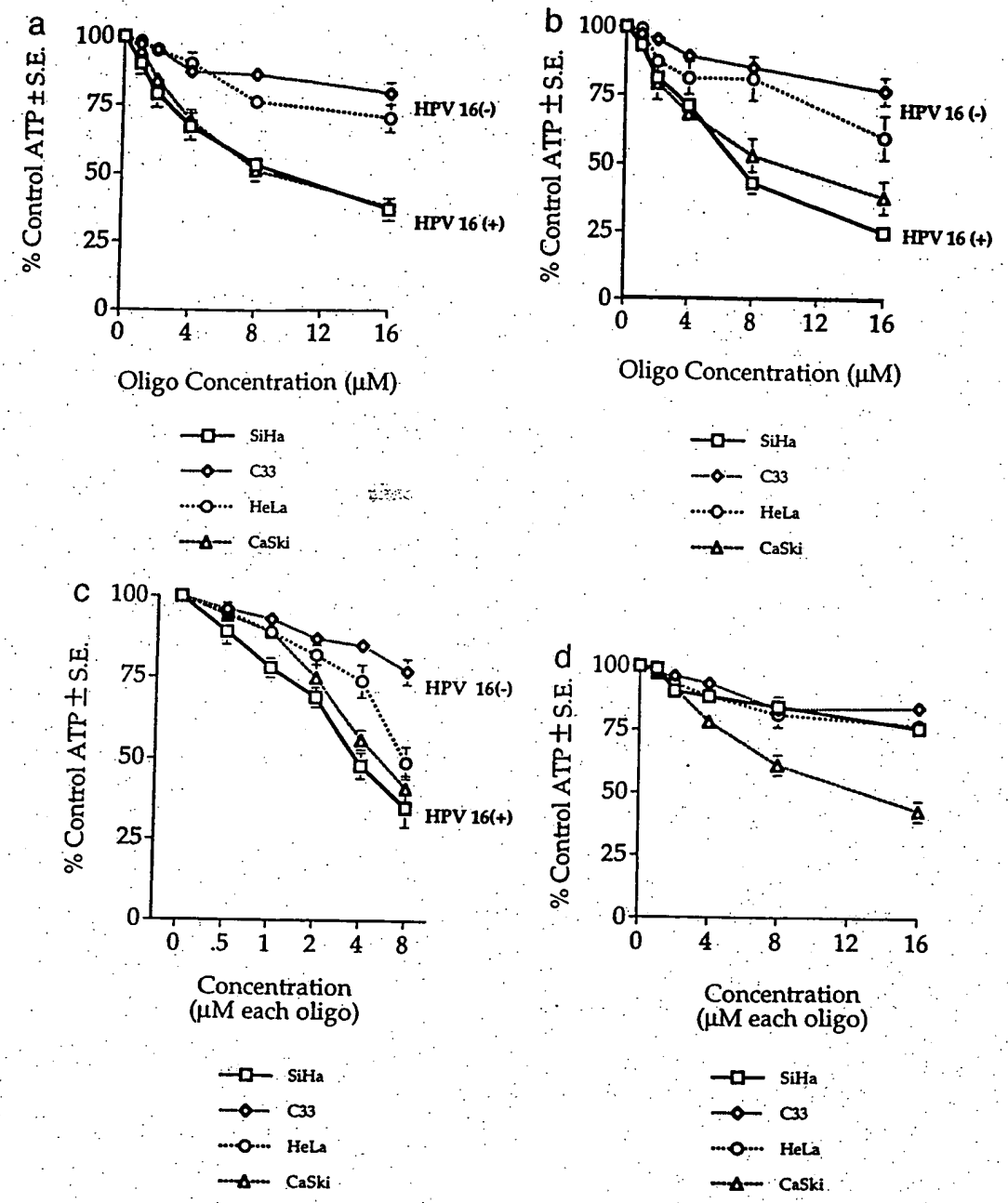

FIG. 1. (a) The reponse of the HPV 16(+) cell lines SiHa and CaSki contrasted with the HPV 16(−) cell lines HeLa and C33 to anti-E6 oligos. Responses clustered into two groups: HPV 16(−) cell lines demonstrating relative resistance to oligos, and HPV 16(+) cell lines exhibiting a steady dose response with >50% inhibition starting at 8 *μM*. (b) The response of HPV 16(+) cell lines SiHa and CaSki, and HPV 16(−) cell lines HeLa and C-33, to anti-E7 oligos at various concentrations.HPV 16(+) cell lines SiHa and CaSki containing the E6-targeted sequence experienced >50% inhibition at oligo doses >8 *μM*. HPV 16(−) cell lines C33 and HeLa remained above 50% inhibition demonstrating minimal oligo effects. (c) The response of HPV 16(+) cell lines SiHa, C33 and HPV 16(−) cell lines to equimolar combinations of anti-E6 and anti-E7 oligos. The dose–response curves for the HPV 16(+) cell lines exhibited inhibition even at very low doses (0.5 *μM*) with increasing levels of inhibition at higher doses. (d) The response of HPV 16(+) cell lines SiHa and CaSki and HPV 16(−) cell lines C33 and HeLa, to a randomized (scrambled) control oligo with similar G*C content to anti-E6 and anti-E7 oligos (see Table 1). Little effect was seen up to 8 *μM* oligo concentrations in all cell lines except CaSki, whose dose–response curve demonstrated increasing inhibition with increasing oligo concentration.

Thus the apparent HPV-16 specificity of HPV-16 E6 and E7 oligos applies to established cell lines as well as fresh cervical carcinoma cells in transient cultures.

To further examine the effects of combined targeting of E6 and E7, CIs were calculated from the median effect parameters as described under Materials and Methods. Con-

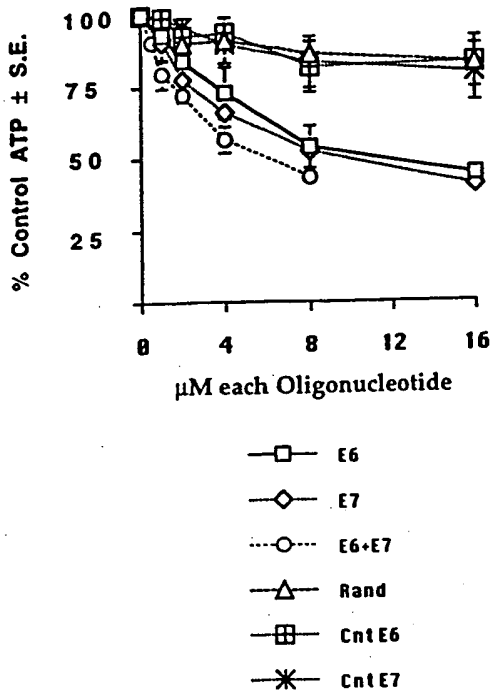

FIG. 2. The response of primary cervical tumors and control endometrial and ovarian tumors to anti-E6 and anti-E7 oligos. Confirmed HPV 16(+) cervical tumors were treated along with three HPV 16(−) controls (two ovarian tumors and an endometrial). HPV 16(+) cervical tumors experienced >50% inhibition at doses of 16 μM, while HPV 16(−) controls show minimal inhibition at similar concentrations.

trary to our initial expectations, E6 and E7 combinations resulted in predominantly antagonistic effects (Fig. 3). Antagonistic effects, however, were either minimal (CaSki) or more pronounced at concentrations of oligos *beyond* the experimental range calculated by median effect parameters. Synergistic effects *did* occur within the experimental range (fraction affected <50%, Fig. 3), but the significance of the *extrapolated* antagonistic effects is unknown.

DISCUSSION

We have previously shown that targeted antigene therapy against c-*myc* and p53 in ovarian cancer cell lines can result in antiproliferative growth effects [19]. Specifically, we have also found that oligos targeting p53 overexpression produce moderate reductions in detectable p53 expression and even more pronounced antiproliferative effects [20]. Interestingly, Storey *et al.* [3] have reported an analogous experience in which oligos targeting HPV-16 ATG start codon sequences produce specific (and nonspecific) growth inhibition in cervical cancer cell lines, even though the rate of synthesis of E6 and E7 proteins and the steady-state levels of E7 remained largely unchanged! They postulated that while specific effects of oligos targeting HPV sequences could readily be demonstrated, the relative absence of changes in protein levels suggests that these oligos may be acting through other, indirect mechanisms, perhaps via interaction with homologous cellular targets. Phosphorothioate oligos have been known to produce nonspecific inhibitory growth effects, predominantly through the nonspecific inhibition of polymerases [21].

While we have not examined alterations in gene product levels in response to E6 and E7 oligos targeting the ATG start codon, we have confirmed the basic findings of Storey *et al.* [3] and Steele *et al.* [4] that antiproliferative effects of E6 and E7 oligos could be tailored to HPV-containing cells. This provides evidence that E6 and E7 sequences play an active role in the maintenance of the malignant growth phenotype and that targeting these sequences may be the basis for future antigene therapies for cervical cancer.

Our data differ from those of Storey *et al.* in two respects. First, they observed inhibitory dose responses to E6 and E7 oligos in the HPV-negative cell line C33 (we did not). Second, their random control oligo produced little effect in the HPV-16-positive cell line CaSki, while we found that this was the only cell line that we tested which produced an inhibitory dose response to a random oligo (albeit moderate). Storey *et al.* speculated that their nonspecific effects might

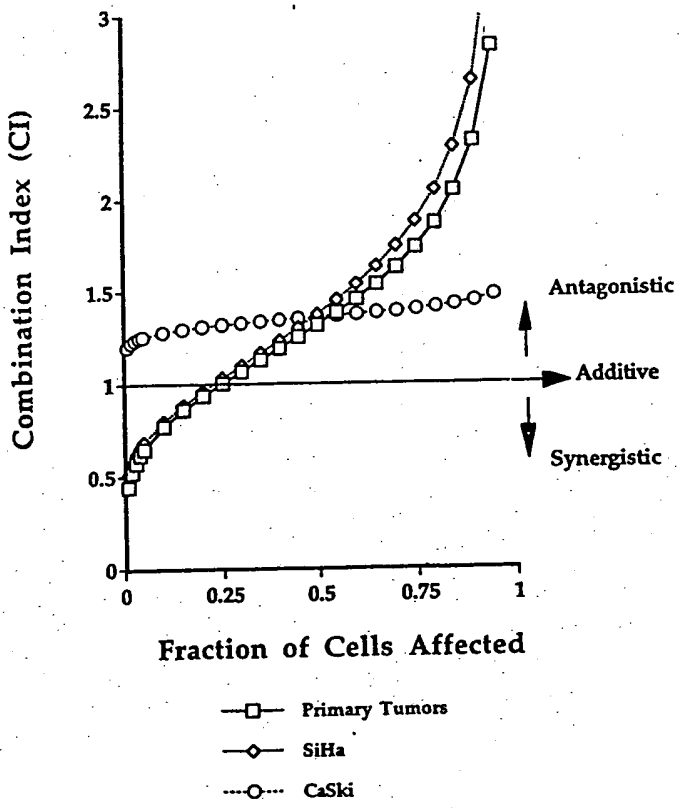

FIG. 3. Combination indices (CIs) calculated from the median effect parameters. Combination of anti-E6 and anti-E7 oligos resulted in predominantly antagonistic effects (CI > 1) at higher doses, while synergism was observed at lower doses (CI < 1).

be due to interaction with the human somatic cytochrome c gene and the c-*jun* protooncogene, which were 78–84% homologous with the HPV oligos [3]. While this possibility is intriguing, however, others have demonstrated that oligos experience a marked degradation in binding ability with even a single base pair mismatch [5]. In contrast to the paper by Tan and Ting, we have not found any significant difference between effects of E6 and E7 in HPV 16-containing cervical cancer cell lines [5, 6]. In fact, with primary cervical tumors, we have observed that anti-E6 oligos have a consistently greater inhibitory effect than anti-E7 oligos. We do not know the reason for this difference in findings, especially in view of the unpredictable oligo responses observed by various other researchers. Perhaps differences in methodologies play an important role in evaluating and comparing the effects of antigene oligos.

Cell line experiments were originally designed with the known HPV 16-negative HeLa and C33-A cell lines. In extending our investigation to primary cervical cell lines, we encountered a series of HPV-16-positive cancers without any evaluable (i.e., uncontaminated, viable in tissue culture) HPV-18 tumors. Analogous to C33-A, we therefore chose to use ovarian and endometrial controls to demonstrate the absence of response in HPV-negative tumors. Due to the prospective nature of acquiring primary advanced cervical tumors, however, we were not able to control the acquisition of HPV-18 primary tumors during the course of this investigation. Early-stage cervical tumors were not studied because of the technical inability in these tumors' tissue cultures (i.e., bacterial contamination). Early-stage cervical cancers tissue samples are usually obtained from septic biopsy specimens usually heavily contaminated, therefore making sterile tumor tissue cultures practically impossible.

The rapid ability to synthesize and test oligos targeting specific gene targets makes them exciting research (and potentially clinical) tools. A number of paradoxes and unexplained phenomena permeate the existing literature, however, and we have attempted to confirm and extend the antigene prospects for oligos targeting HPV sequences. We have generally confirmed the specific activity of these oligos in HPV-16-positive cells, and extended these findings to primary cervical explants derived from advanced, metastatic lesions. Although we have previously found combination antigene therapy to yield synergistic effects [19], combined targeting of E6 and E7 in this study unexpectedly produced mixed synergistic and antagonistic effects. Although this finding is difficult to explain, one possible mechanisms may involve interference of one oligo by the other, such as through nonspecific binding. The nucleotides 18–22 of anti-E6 oligos may bind to nucleotides 6–10 of anti-E7 oligos, thereby causing such an effect. Even weak or nonspecific binding of one oligo to another may decrease the antiproliferative effects to the point of causing an antagonistic net effect of the combination E6/E7 oligos.

Before the application of antigene oligo technology to the clinical setting for cervical cancer therapy, further *in vitro* studies are needed to determine the reliability and feasibility of this approach.

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CANCER LETTERS

Immortalization of human adult prostatic adenocarcinoma cells by human papilloma virus HPV16 and -18 DNA

Z. Naghashfar[a], J.A. DiPaolo[b], C.D. Woodworth[b], A. Passaniti[a,*]

[a]*Laboratory of Biological Chemistry, Gerontology Research Center, National Institute on Aging, National Institutes of Health, 4940 Eastern Avenue, Baltimore, MD 21224, USA*

[b]*Laboratory of Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA*

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Abstract

Primary prostate epithelial and prostate adenocarcinoma cells cultured in serum-free medium grew for up to 10 passages before senescence. Cells from prostate adenocarcinoma of a 55-year-old patient without lymph node involvement were transfected with plasmids containing recombinant human papilloma virus HPV16 or HPV18 DNA and the selectable neomycin-resistance gene. After G-418 selection, cells underwent crisis, and surviving cells infected with retroviruses encoding the HPV18 E6/E7 genes (HPV-PAC1), transfected with a head-to-tail dimer of the complete HPV16 genome (HPV-PAC2), or transfected with HPV18 E6/E7 early genes (HPV-PAC3) were established. HPV-PAC1 and HPV-PAC2 cultures appeared morphologically similar to primary cultures even after 40 passages. However, HPV-PAC2 cultures had a clonal morphology. All lines were positive for cytokeratin 18, had acquired vimentin expression, and contained either HPV16 or HPV18 sequences integrated into host DNA. None was tumorigenic in nude mice or formed colonies in soft agar. These cells did not secrete prostate specific antigen nor respond to androgen although tamoxifen inhibited the growth of the cells. Immunohistochemistry showed no evidence of p53 overexpression. Further characterization of these cell lines and examination of their response to chemotherapeutic agents may provide relevant information for the study of hormone-independent PC.

Keywords: Human prostatic cell; Immortalization; Human papilloma virus DNA

1. Introduction

Prostatic carcinoma is the most frequently diagnosed malignancy and a major cause of cancer deaths for males in the United States second only to lung cancer [1]. The incidence of prostate carcinoma increases most rapidly with age [2,3]. Although potentially curable in its early stages, metastatic prostatic cancer is incurable, with temporary remission commonly achieved by hormonal therapy. Despite the high morbidity and mortality of the disease, the molecular events involved in the development and progression of prostate cancer are still poorly defined. Mutational activation of the ras oncogenes [4] and inactivation of the p53 and retinoblastoma tumor suppressor genes [5–7] have been observed. The frequency of these genetic events, however, appears to be rather low, even in advanced stages of the disease.

* Corresponding author.

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Therefore, a growing need to gain further understanding of the genetic pathways underlying prostate tumorigenesis exists.

Epidemiological and clinical studies implicate human papilloma virus (HPV) in the etiology of a variety of squamous epithelial tumors [8,9]. HPV16 and -18 account for approximately 70% of HPV positive cervical carcinoma. Unlike DNA from HPV-6 and -11, which induce benign genital tumors [10,11], DNA from HPV16 and -18 can immortalize primary keratinocytes in culture and this difference in transformation ability appears to be due to biological differences in their E6 and E7 genes [12,13]. The E6 and E7 proteins of malignancy-associated HPV but not benign-associated HPV combine efficiently with the tumor suppressor proteins of the p53 and retinoblastoma genes, respectively [14,15]. Differences also exist between HPV16 and HPV18 in that the latter is 10–50-fold more active in immortalizing keratinocytes in vitro [16], and these differences map predominantly to the viral enhancer region [17]. Reports show that HPV18-immortalized keratinocytes spontaneously progressed to malignancy [18].

One of the most characteristic features of HPV infectivity is site and cell type specificity. The current evidence does not implicate HPV in human prostate cancer. While earlier studies claimed a high prevalence of HPV16 in benign prostatic hyperplasia (BPH) and prostate adenocarcinoma [19–21], more recent evidence strongly argues against any causative involvement of HPV in human prostate tumorigenesis [4,22,23]. HPV16 and 18 DNA was used in the present study to immortalize human prostate epithelial (HPE) cells to provide a model for investigating the pathways in prostate tumor progression.

2. Materials and methods

2.1. Prostate cell culture

Human prostatic tissue was obtained after prostatectomy from patients diagnosed with primary adenocarcinoma. Tissue was minced to 1–2 mm and incubated ON at 37°C in collagenase 200 U/ml (Worthington) in HBSS, with 5% FBS. The digest was triturated by repeated pipetting and centrifuged. The pellet was washed with HBSS, resuspended and plated in 100 mm culture dishes. The culture media was a modification of MCDB153-LB [24]. When prostate epithelial cell colonies reached 0.5–1 cm^2, the primary culture was passaged with trypsin/EDTA (0.2%/0.02%). Cells were plated at a density of approximately 0.5×10^6 in 100 mm dishes and used for HPV transfection.

2.2. Recombinant plasmids

The plasmid pMHPV16d, a head to tail dimer of HPV16 DNA [25] inserted into the BamHI site of the plasmid pdMMTneo [26] and recombinant retroviruses encoding HPV18 E6 and E7 genes have been described [27,28].

2.3. DNA and RNA analysis

High molecular weight DNA was isolated using DNA Stat-60 (Tel-test B, Inc.). Ten micrograms of purified DNA was digested with restriction endonucleases BamHI or EcoRI and DNA was separated by electrophoresis in 0.8% agarose gels and transferred to Gene Screen membrane (New England Nuclear).

DNA probes used for hybridization analysis were the BamHI fragment of HPV16 or the EcoRI fragment of HPV18, purified from the respective vectors. DNA was labeled to high specific activity (5×10^8 dpm/μg of DNA) by using [^{32}P]dCTP and the multiprimer DNA labeling kit purchased from Amersham (Amersham Corp., Arlington Heights, IL). Membranes were hybridized according to the protocol described by Church and Gilbert [29]. Prehybridization was performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA and 1% bovine serum albumin for 2–4 h at 65°C in rotating tubes. Hybridization was performed overnight in the same buffer. The filters were washed first with 2× SSC, 0.5% SDS, then with 2× SSC, 0.1% SDS at 24°C for 15 min and finally with 0.1× SSC and 0.5% SDS at 65°C for 2 h (Tm-17C). Under these conditions HPV16 and -18 do not cross hybridize. Autoradiography was performed by exposing the filters to Kodak XAR-5 film at –70°C with an intensifying screen for 24 h. To estimate HPV copy number, an amount of plasmid equal to 0.5, 2.5 or 5 copies per cell was mixed with salmon sperm DNA and also analyzed by Southern blot. The 8 kb position indicates linear HPV.

Total RNA was extracted from the cells with RNA State-60 (Tel-test B, Inc.). RNA electrophoresis was performed in 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to a Gene-Screen filter and hybridized as described for DNA.

2.4. Immunohistochemistry

Cells were fixed in culture dishes with ice-cold methanol/acetone (2:1) for 15 min, air-dried and used for immunostaining. The antigens tested were identified by indirect immunofluorescence or immunoperoxidase labeling. Mouse monoclonal antibodies were used to detect cytokeratin 18 (Sigma), Vimentin (Sigma), prostatic specific antigen PAS (Sigma), prostatic acid phosphatase, PAP (Sigma), and p53 (Oncogene Science). After 1 h incubation at 24°C with the appropriate dilution of cytokeratin antibodies, rabbit anti-mouse immunoglobulin was added for 30 min.

2.5. Growth curves and androgen binding

The effect of the anti-estrogen tamoxifen on HPE growth was determined on cultured cells 24 h after attachment. The medium was replaced with medium containing 0.01, 0.1, 1, or 10 μM tamoxifen. Triplicate cultures were incubated for 7 days and medium was changed every 48 h. Cells were trypsinized and counted with a hemacytometer. Viability was determined with 0.4% Trypan blue. To determine the presence of androgen receptors, intact cells were assayed for specific binding of the synthetic androgen, [^{3}H]mibolerone (New England Nuclear). The cells were cultured in 12-well culture plates in SFM-keratinocyte basal medium for 72 h. Medium containing labeled mibolerone (10^{-12}–10^{-7} M) was added for 1 h at 37°C. Intracellular radioactivity was solubilized with 0.5 M NaOH after washing the wells three times with ice-cold TCA. For growth assays with mibolerone (10^{-12}–10^{-7} M) the total number of cells were counted after 7 days.

3. Results

Primary cultures of HPE cells derived from normal prostate tissue migrated from attached acini (Fig. 1, passage 0) and reached confluence within 1 week (Fig. 1, passage 1). With subsequent passages, the cells became larger and more flattened (Fig. 1, passage 5), appeared to senesce, and could no longer be subcultured.

Primary cultures from prostate tissue of a patient diagnosed with prostatic adenocarcinoma (Gleason

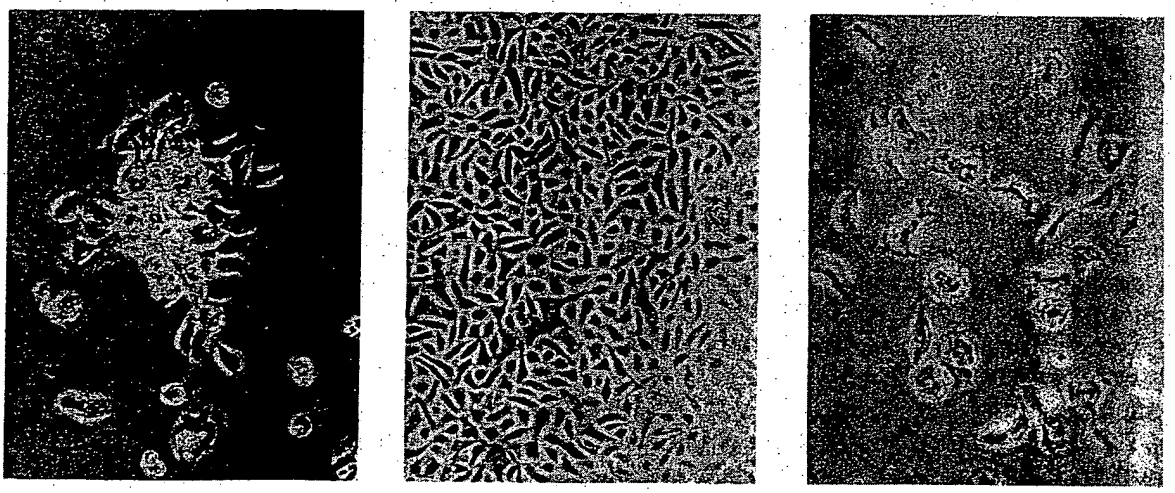

Fig. 1. Morphology of primary HPE cultures. Acinar structures 1 day after collagenase digestion of primary prostate tissue (passage 0). Confluent monolayer of epithelial cells 1 week after isolation (passage 1). Senescent cells (passage 5).

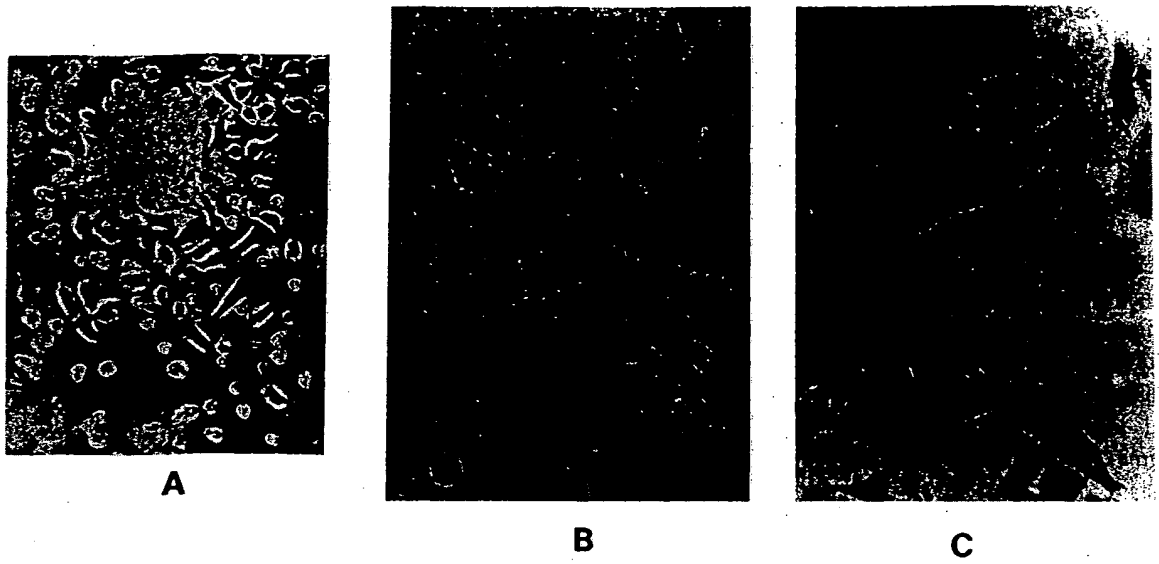

Fig. 2. Morphology of HPV transfected prostate adenocarcinoma cells. (A) Cells migrating from isolated acini. (B) HPV16d immortalized cells post-crisis exhibit rounded morphology. Cells are stained with keratin antibody. (C) HPV18 E6, E7 DNA immortalized cells exhibit a typical epithelial morphology (keratin stained). No specific staining was observed without primary antibody and in control fibroblast cultures.

grade 3 + 4 = 7) migrated from attached acini (Fig. 2A) and formed typical epithelial monolayers similar to HPE cells from normal prostate. These cells also eventually senesced (data not shown). Secondary cultures of these adenocarcinoma cells were transfected with pMHPV16d, the HPV18 E6/E7 genes or vector pdMMTneo or infected with the retrovirus HPV18 E6/E7 genes [28] and selected for resistance to the antibiotic G418 (100 μg/ml of media) for 48 h. Cells were passaged by trypsinization once a week. Cells transfected with HPV16d continued to proliferate after passage 10 followed by a series of crises after passage 25. After 3 months, significant differences in morphology were observed (Fig 2B). The cells were smaller and almost round. Growth during crisis was very slow with doubling times of 60–90 h but after crisis (passage 68) the doubling time was 40 h. The cells have been subcultured repeatedly and no further crises have been observed. In contrast, HPV18 transfected cells did not undergo crisis with subculturing. Several HPE isolates were immortalized by HPV18 DNA, one of which is described here (Fig. 2C). These HPE/HPV immortalized cell lines grew to confluence but did not overgrow the mono layer and thus never lost contact inhibition. No colony formation in soft agar was observed, nor were tumors induced after subcutaneous cell injection into nude mice. The effect of tamoxifen on cell growth was examined because of the reported effects of tamoxifen in increasing TGFβ production. Tamoxifen inhibited the growth of HPE/HPV cells at concentrations greater than 1 μM (data not shown).

HPE/HPV cells grew in SFM-keratinocyte medium and exogenous androgen did not stimulate the growth of the cells suggesting a loss of androgen response. Confirming this, specific hormone binding assays were negative. Because PSA expression is regulated by the androgen receptor, the expression of PSA and specific epithelial cytokeratin 18, vimentin, and prostatic acid phosphatase (PAP) were examined by immunocytochemistry (Table 1). Cytokeratin 18 is constitutively expressed in secretory prostatic epithelial cells and was detected in HPE/HPV 16 and 18 immortalized cells. Vimentin which is often present in cultured epithelial cells, was also present. PAP and PSA, markers of secretory prostatic cells in normal and well differentiated prostatic carcinoma, were present in the initial passages, but progressively di-

minished and were not detectable after passage 10. At passage 35 PSA was negative when analyzed by ELISA techniques (Tandem PSA kit, Hybritech Corp) which can detect 1 ng/ml of PSA. p53 protein expression was negative as expected because the E6 protein of oncogenic HPV types binds to p53 leading to increased protein turnover.

To determine whether HPV16 or 18 DNA was present in the HPV16 or 18 immortalized HPE cell lines, high-molecular-weight DNA was obtained at early (E) or late passages (L) (12–14 to 35–50). Digestion with EcoRI and detection with an HPV18-specific probe revealed a band from HPE/HPV18 transfected cells and control HeLa cells (Fig. 3A), indicating integration of the HPV genome. HPE/HPV16d transfected cells and control SiHa cells (containing HPV16) did not exhibit a band. However, an HPV16-specific probe detected HPV16 sequences in HPE/HPV16d transfected early and late passage cells and control SiHa cells. The HPE/HPV16d cell line was tested for resistance to G418, and results revealed loss of resistance to G418 while the HPE/HPV18 cell line retained G418 resistance.

To estimate the number of HPV 16 copies in the transfected lines, a reconstruction experiment was performed by mixing amounts of the plasmid DNA equivalent to 0.5, 2.5 and 5 HPV copies per cell with salmon sperm DNA. The DNA was digested with BamHI. The copy number of HPV in the HPE/HPV cell lines was less than 1 suggesting that part of the HPV genome was lost. The state of the HPV16 sequence in HPE/HPV16d cell lines at early and late passages was similar and suggests that rearrangements did not occur during culture.

Table 1
Characteristics of HPV immortalized prostate cells

	HPE	HPE/HPV18	HPE/HPV16d
Cytokeratin 18	+	+	+
Vimentin	n.d.	+	+
PSA	±	–	–
PAP	n.d.	–	–
Androgen receptor	n.d.	–	–
Soft agar growth	–	–	–
Tumors nude mice	–	–	–
p53	n.d.	–	–

n.d., not done.

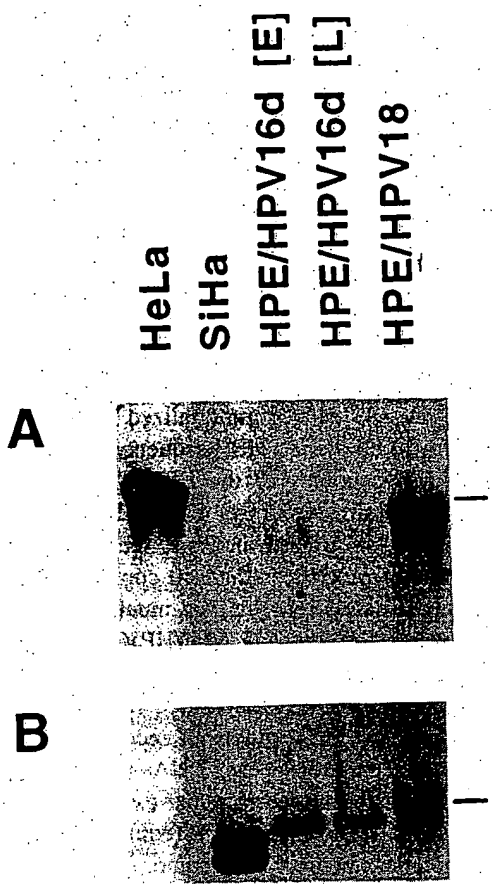

Fig. 3. Southern blot to detect HPV18 and HPV16 sequences. Genomic DNA was digested with EcoRI and resolved on agarose gels. (A) An HPV18-specific probe was used. HeLa cell DNA was the positive control. (B) An HPV16-specific probe was used. SiHa cell DNA was the positive control. An 8 kb BamHI plasmid fragment was used as a marker and is indicated by the bar.

4. Discussion

Primary adenocarcinoma prostate cells were immortalized using HPV16 and 18 DNA. The transfected HPE/HPV cell lines have now been cultured for more than 12 months and 70 passages. HPV16 or 18 DNA was responsible for the extended life-span of HPE cells since vector sequences alone were ineffective. Maintenance of HPV sequences did not depend on G418 expression as demonstrated by two of the three cell lines that lost G418 resistance.

None of the primary prostate epithelial cell isolates were immortalized with the recombinant papillomavirus DNA. In contrast, the efficiency of immor-

talization of keratinocytes is higher. This may be due to the fact that in cervix the relatively abundant basal cells are susceptible to infection by virus, and as a result of differentiation clones of infected cells can be detected by in situ hybridization. The low efficiency in prostate tissue may result from (1) fewer basal cells in the prostate, compared to the cervix, (2) the medium used in these experiments which was optimized for growth of keratinocytes and not prostate epithelial cells, or (3) the age of the donor.

HPV sequences are found in the overwhelming majority of cervical carcinoma or carcinoma-derived cell lines [30–33]. The immortalized HPE cells in this study also contained HPV sequences. HeLa cells contain 50–100 copies of the HPV18 genome and SiHa cells contain one copy of the HPV16 genome [34]. From the Southern analysis, the HPE/HPV18 cells contained much less than 50 copies of HPV18 since approximately one-half as much HeLa DNA was applied to the gel than HPE/HPV18 DNA. The HPE/HPV16 cells contained less than one copy of the HPV16 genome per cell as verified from the Southern analysis (Fig. 3B) and from reconstruction experiments with salmon sperm DNA. Our results are consistent with the hypothesis that extension of lifespan (immortalization) by HPV requires integration of the viral DNA in the host genome.

The available human prostate carcinoma cell lines PC-3 [35], DU-145 [36], TSU-pr1 [37], and LNCaP [38], are derived from metastases, and only LNCaP is androgen dependent. This cell line has a mutation at codon 877 in the androgen receptor gene, a mutation frequently seen in treatment-resistant prostate carcinoma. To study the events leading to prostate tumorigenesis, several groups have cultured immortalized prostate epithelial cells. Viral immortalization using SV40 T antigen, adenovirus E1A, or HPV18 DNA have been reported [21,39–41]. Adenovirus E1A was unsuccessful (W. Isaacs, pers. commun.). We also found HPV18 to be effective at immortalization. In addition, although HPV16 may be less oncogenic than HPV18, the current results show for the first time that the HPV16 dimer can also immortalize human prostate epithelial cells.

In humans, the standard therapy for metastatic prostatic cancer has been androgen ablation. Androgen independent prostate cancer cells, however, exist even before therapy is initiated. In animal models androgen ablation induced by castration causes the regression of tumor due to activation of apoptosis of androgen dependent cells [42]. The effect is probably due to an enhanced expression of the transforming factor beta (TGFβ) gene which is also a potent inhibitor of cell proliferation of many malignant epithelial cells. The HPV immortalized cell lines were sensitive to the growth inhibitory properties of tamoxifen which may be mediated by TGFβ. Tamoxifen reduced the growth of these cell lines by 90% while TGFβ reduced cell growth by 30%. New therapeutic approaches for androgen independent prostate cancer leading to a programmed cell death pathway are required. The use of agents that activate TGFβ in such therapy needs further investigation.

Effective chemotherapy specifically targeted against the androgen-independent cancer cell can be combined with other methods. Unfortunately there are presently no highly effective chemotherapeutic agents that can eliminate androgen-independent prostatic cells. HPE/HPV immortalized cell lines combined with other prostatic metastatic cell lines may be useful in examining the effects of various therapeutic agents that may be effective inhibitors of androgen-independent prostate tumor cells.

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Survey

Control of interferon signaling in human papillomavirus infection

Antonis E. Koromilas* [a,b,c,d,e,1], Suiyang Li [a], Gregory Matlashewski* [d,2]

[a] *Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, 3755 Cote-Ste.-Catherine Road, Montréal, Québec, Canada H3T 1E2*

[b] *Department of Oncology, McGill University, Montréal, Québec, Canada*

[c] *Department of Medicine, McGill University, Montréal, Québec, Canada*

[d] *Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada*

[e] *Department of Cell Biology and Anatomy, McGill University, Montréal, Québec, Canada*

Abstract

Human papillomaviruses (HPV) infect mucoal and cutaneous epithelium resulting in several types of pathologies, most notably, cervical cancer. Persistent infection with sexually transmitted oncogenic HPV types represents the major risk factor for the development of cervical cancer. The development of HPV-associated cervical cancer has been closely linked to the expression of the viral oncogenes E6 and E7 in the tumor cells. The major viral oncoproteins, E6 and E7, target the cellular tumor suppressor gene products p53 and Rb, respectively. As detailed within, these interactions result in the stimulation of proliferation and the inhibition of apoptosis, thus representing major oncogenic insults to the infected cell. In addition to mediating transformation, the E6 and E7 genes also play significant roles in altering the immune response against infected cells by suppressing interferon (IFN) expression and signaling. At the clinical level, IFNs have been used in the treatment of HPV-associated cervical intraepithelial neoplasia (CIN) or cervical cancers with mixed results. The success of the treatment is largely dependent on the subtype of HPV and the immune response of the patients. Despite this inefficiency, the increasing knowledge about the regulation of IFN signaling pathways at molecular level may hold a promise for the use of new therapeutic strategies against HPV infection. Studies on the regulation of the function of IFN-inducible gene products by the E6 and E7 may lead to the development of new therapeutic approaches based on strategies that modify the function of the HPV oncoproteins and restore IFN-signaling pathways through endogenous control mechanisms.

Contents

E-mail addresses: akoromil@ldi.jgh.mcgill.ca (A.E. Koromilas), greg_matlashewski@maclan.mcgill.ca (G. Matlashewski).

[1] Corresponding author. Tel.: +514-340-8260, ext.3697; fax: +514-3407576

[2] * Corresponding author. Tel.: +1-514-398-3914; fax: +1-514-398-7052.

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1. Human papillomaviruses

Human papillomaviruses (HPVs) are small double stranded DNA viruses, which infect cutaneous and mucosal epithelium resulting in a variety of clinically important conditions, most notable cervical cancer [1]. The incidence of cervical cancer in North America is relatively uncommon due to the effectiveness of screening programs that assess cervical cytology by the Papanicolaou smear. However, on a global scale, cervical cancer is a major cause of mortality, especially in developing countries where screening programs are not routinely performed [2]. Precursor lesions for cervical cancer are known as cervical intraepithelial neoplasia (CIN). The observation that CIN occurs at a younger age than does invasive disease is consistent with the long latency period required to develop cervical cancer, which can take over 10 yr. Therefore, cervical cancer is a preventable disease if CIN lesions are detected early.

The epidemiology of cervical cancer has always suggested that it is sexually transmitted disease [3–5] implying an etiological role for an infectious agent. There is now no doubt that the infectious agent involved in cervical neoplasia is HPV and infection with the so-called oncogenic or high risk HPV types 16, 18, 31, 33, 35, 39, 41–45 is the major risk factor for this disease. However, the available epidemiological evidence also shows that that HPV infection alone is not sufficient for neoplastic progression. Thus only a fraction of individuals infected with high-risk viruses go on to develop CIN or cervical cancer. About 30% of CIN cases will progress to cervical cancer [6]. The HPV genome is usually present in an episomal configuration during infection and CIN, whereas in invasive cervical cancer, the genome is commonly integrated into the host DNA [7]. The only viral genes which are consistently expressed following integration is the E6 and E7 oncogenes and these genes are critical for the development of malignant transformation and also play a role in altering the cellular response to cytokines.

2. Transforming activity of the HPV oncoproteins

The genome of HPVs contains eight genes including the E6, E7, E5, E4, E2, E1, L1 and L2 genes. The early (E) genes are predominantly involved in regulating the viral life cycle and the late (L) genes encode the viral capsid proteins. The major viral transforming genes are the E6 and E7 genes, which target the cellular p53 and Rb genes respectively [8–10]. This review will therefore focus on the biological activity of the E6 and E7 genes.

The oncogenic potential of the E6 and E7 proteins from the high-risk HPVs is due predominantly to the ability of these viral proteins to target and inhibit the activity of the cellular p53 and Rb tumor suppressor proteins respectively. Since p53 and Rb induce apoptosis and control cell-cycle progression respectively, their inactivation by E6 and E7 represents a major carcinogenic insult to the infected cell. The continued expression of E6/E7 is necessary to sustain the proliferation and anti-apoptotic character of cervical cancer derived cells [11–13]. The ability of HPV to target both Rb and p53 allows the virus to stimulate cell replication, which also results in the replication of the viral genome while inhibiting apoptosis in the infected cell. Moreover, it has also been revealed that E6 and E7 may play a role in maintaining viral episomal DNA stability during infection [14].

3. The transforming activity of E6 and inhibition of apoptosis

The E6 proteins contain approximately 150 amino acids depending on the HPV type with an apparent molecular weight of about 18 kDa. Due to the low levels of this protein in the cell and a lack of good antibodies, it has been difficult to determine its location within the cell. Our own recent observations show that the oncogenic E6 proteins are equally distributed between the nucleus and the cytoplasm, whereas E6 from the non-oncogenic HPV types are predominantly cyto-

plasmic (unpublished observations). This is consistent to what has been previously reported for the oncogenic HPV E6 proteins [15,16]. It is also possible that the location of the E6 protein could change depending on the differentiation status of the cell or in response to different extracellular stimuli.

As mentioned above, a major target of oncogenic type E6 is the p53 tumor suppressor protein and this results in the degradation of p53 via the ubiquitin proteolytic pathway [17,18]. This observation provides a logical explanation why the p53 tumor suppressor gene is seldom mutated in HPV-positive cervical tumors [19,20]. In comparison, the p53 gene is mutated in the majority of other cancer types [21]. Cells expressing E6 lose the G1 checkpoint presumably due to loss of p53 [22] and are resistant to p53-mediated apoptosis [23–25]. The degradation of p53 by oncogenic type E6 is dependent upon a cellular protein termed E6-AP (E6-Associated Protein) [18,26]. There has however not been any link demonstrated to date between E6-AP and p53 in the absence of E6.

The ability of E6 to mediate p53 degradation is very important considering that p53 places a zero tolerance on cellular abnormalities through mediating apoptosis and preventing cell proliferation [27,28]. Therefore in order for tumor cells or viral oncogene expressing cells to survive, the p53 tumor suppressor protein must be lost through gene mutation or viral protein interaction. In the case of oncogenic HPVs, E6 is responsible for mediating the degradation of p53 thus ensuring the survival of the infected cell [14].

Another important activity of E6 with respect to mediating cellular immortalization and subsequent transformation is its ability to activate cellular telomerase activity [29]. The telomerase enzyme is important to maintain the stability of the chromosome ends (telomeres) during multiple rounds of cell proliferation. It is however unclear what role the telomerase enzyme may play in the normal virus life cycle.

A number of other cellular targets for E6 have also been identified and these may also contribute to the oncogenic activity of this protein. For example, the E6/E6-AP complex has also been implicated in the degradation of the apoptosis inducing c-Myc [30] and Bak proteins [31]. E6 from HPV-16 can also associate with the transcriptional co-activators CBP and p300 [32,33]. Since CBP/p300 is involved in the transcriptional activity of p53, this association also reduces the transcriptional transactivation activity of p53. Oncogenic HPV E6 has also been shown to mediate the degradation of the discs large (Dlg) tumour suppressor proteins, which have been shown to have tumor suppressor protein activity in *Drosophila* [34]. Dlg proteins may function as components of signal transduction pathways transmitting growth inhibitory signals from regions of cell–cell contact to downstream effectors which block cell proliferation and migration [35]. Taken together, E6 may have multiple activities in addition to inhibiting p53 and further studies are needed to define these interactions with respect to cell transformation.

4. The transforming activity of E7 and induction of cell cycle progression

As with E6, expression of E7 is necessary for the continuing proliferation of HPV containing cervical cancer cell lines [11,36,37]. The E7 proteins from the various HPV types contain approximately 100 amino acids and, similar to E6, contain a zinc binding motif in the C-terminal region [38,39]. The E7 protein is normally phosphorylated by casein kinase II [40] and has been located in a variety of cellular locations including the cytoplasm, the nucleus, and the nucleoli [41–44]. The HPV E7 shares homology with other DNA tumor virus proteins including Adenovirus E1A and SV40 large T and has been shown to interact with many of the same cellular targets including the Rb, p107, and p130, cyclin A, cyclin E, and AP-1 [44–48]. Given the nature of these cellular targets, these interactions suggest that a major role for E7 is to disrupt normal cell cycle control. Several studies have shown that expression of E7 is associated with increased cellular DNA synthesis [49–51]. The increase in DNA synthesis and cell proliferation is largely as a result of the interaction between E7 and the Rb family of proteins including Rb, p107 and p130. These interactions interfere with the ability of the Rb family to suppress the transactivating activity of the E2F transcriptional transactivators. E2F responsive promoters include dihydrofolate reductase, thymidine kinase, cdc 2, cyclin A, cyclin D, and DNA polymerases [52]. Expression of these genes is required for DNA synthesis and progression through the cell cycle and expression of E2F alone has been shown to induce DNA synthesis [53]. Therefore, E7 perturbs the normal control of cell proliferation by inhibiting the function of Rb resulting in the activation of E2F mediated transcription resulting in the induction of gene products which stimulate DNA synthesis and cell proliferation. It has also been shown that E7 destabilizes Rb and the related p107 protein potentially through the ubiquitin proteasome pathway [54,55]. The crystal structure of a C-terminal region 9 amino acid E7 peptide bound to Rb has now been defined [56].

In addition to targeting Rb and related proteins, E7 has also been shown to interact with the other cell cycle inhibitors including p21 [57,58] and p27 [59]. Clearly the interaction and suppression of these cell cycle inhibitors contribute further to the ability of E7 to stimulate cell proliferation through disrupting key cell cycle control molecules.

5. Synergism between E6 and E7 in inducing cell proliferation and inhibition of apoptosis

As mentioned above, both E6 and E7 bind to a number of cellular proteins, which play key roles in regulating cell cycle and apoptosis. It is noteworthy that inactivation of Rb results in the induction of E2F-mediated transcription, which in turn leads to the expression of the p14 ARF protein that stabilizes p53 by inhibiting MDM2-mediated p53 degradation [28]. Consequently, expression of oncogenes with E7-like activity can effectively induce p53-mediated apoptosis. Therefore, it is essential that HPV counteract the E7-mediated apoptosis through the above pathway by targeting p53 for degradation with the E6 protein. A summary of the functions of E7 and E6 in inducing cell proliferation and inhibiting apoptosis is shown in Fig. 1. Under these conditions, the viral genome is thus allowed to replicate and ultimately produce new viral particles in infected terminally differentiated cells.

6. HPV infection and regulation of immune system

Persistence of cutaneous and genital-HPV-induced lesions is common suggesting that infections can be sustained for an extended period without the induction of an effective immune response [60]. This also suggests that HPVs have evolved mechanisms for subverting the activation of immune response. Limiting exposure of viral gene products to the host immune system is an important evasion mechanism for these viruses [61]. First, there is no viremic phase during the life cycle, so the systemic immune system is avoided. Second, very low levels of viral proteins are expressed in the basal and spinous layers of epidermis, where they would be most likely to be recognized by Langerhans cells and infiltrating lymphocytes. Thirdly, extensive production of viral proteins takes place in terminally differentiated layers of the epidermis where shedding of the virus from this surface limits the exposure of assembled virions to the host immune system.

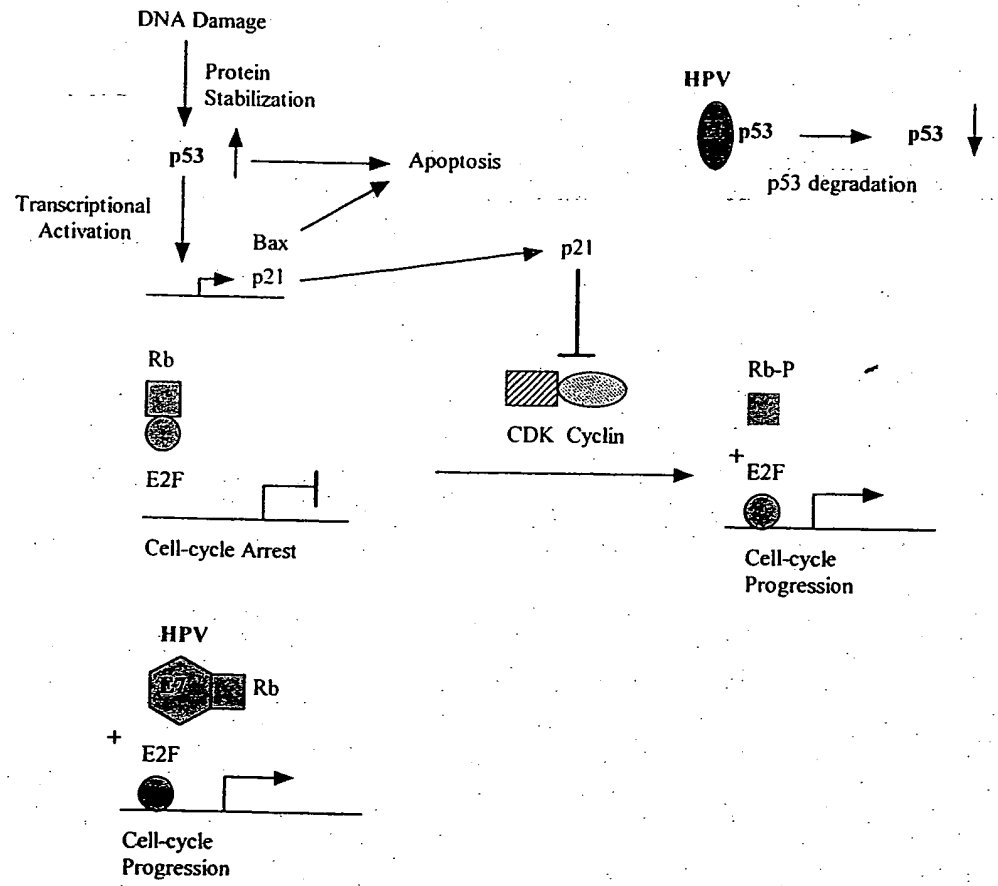

Fig. 1. Biochemical and biological functions of E6 and E7 proteins: DNA damage leads to phosphorylation and stabilization of p53. Activated p53 induces the transcription of genes involved in cell cycle arrest (e.g. the CDK inhibitor p21) or apoptosis (e.g. Bax). The E6 oncoprotein blocks p53 activation by degradation through the proteasome pathway. The E7 oncoprotein binds to the hypophosphorylated form of Rb in the so-called "pocket domain", which is partially conserved among the other Rb-related proteins. The effect of this interaction is to release free the transcription factor E2F, which is now able to activate the transcription of genes required in G1/S cell-cycle progression.

Table 1
Efficacy of IFNs in the treatment of HPV-associated disease

Disease	HPV subtype	Efficacy	References
IFN-α			
Cervical intraepithelial neoplasia			
CIN of lower genital tract	16	Effective	[99]
CIN	6/11/16/18	Partial	[100]
Cervical cancer	N/A	Partial	[101]
CIN	16	Partial	[102]
HPV-infected cervix	N/A	Partial	[103]
CIN2	N/A	No effect	[104]
Other genital infections			
Condylomata acuminata	6/11	Effective	[105]
Buschke-Lowenstein tumor	6/11	Effective	[106]
Vulvar vestibulitis	16/18	Effective	[107]
Condylomata acuminata	6	Effective	[108]
Intravaginal warts	N/A	Effective	[109]
Infected lower genital tract	N/A	Effective	[110]
Genital condyloma acuminatum	N/A	Effective	[111]
Genital warts	N/A	Effective	[112]
Chronic vulvodynia	N/A	Partial	[113]
Vestibular papillomatosis	N/A	Partial	[114]
Cervical condyloma	11/16	Partial	[115]
Genital warts	16/18	Partial	[116]
Condylomata acuminata	16/18	Partial	[117]
Microcondylomatosis	N/A	Partial	[118]
Genital HPV-infections	6/11/16/18	No effect	[119]
Laryngeal lesion			
Laryngotracheal papillomatosis	6/11	Effective	[120]
Laryngeal papilloma	6	No effect	[121]
Respiratory tract papillomas			
Respiratory tract papillomas	6/11	No effect	[122]
IFN-β			
Cervical intraepithelial neoplasia			
CIN grade II	N/A	Effective	[123]
CIN	N/A	Effective	[124]
CIN-II	N/A	Effective	[125]
CIN	N/A	Effective	[126]
CIN I, II & III	N/A	Effective	[127]
CIN	N/A	Effective	[128]
CIN	N/A	Partial	[129]
CIN I & II	N/A	Partial	[130]
Other genital infections			
Genital condylomata acuminata	N/A	Effective	[131]
Intraepithelial genital lesion	N/A	Effective	[132]
Genital HPV infections	N/A	Effective	[133]
Severe vulvar vestibulitis	N/A	Effective	[134]
Cervical & vaginal lesions	N/A	Effective	[135]
Genital condylomatosis	N/A	Effective	[136]
Genital warts	2/6/11	Partial	[137]
HPV-infected dysplasia	N/A	Partial	[138]
Lower female genital tract	N/A	Partial	[128]
Vulva infection	N/A	No effect	[139]
IFN-γ			
Cervical Intraepithelial neoplasia			
CIN	High risk	Effective	[140]
Cervical carcinoma	16	Effective	[141]
Other genital infections			
Condylomata acuminata	6	Effective	[142]
Bowenoid papulosis (BP)	16	Effective	[143]
Genital warts	All types	Effective	[144]

The expression of major histocompatibility complex (MHC) class I- and MHC class II-restricted T-cell responses are important for the control of most viral infections [62]. However, it appears that HPVs have evolved specific mechanisms to prevent their proteins, particularly those expressed in the lower layers of the epithelium, from being efficiently recognized by T cells. This is possibly mediated by producing viral proteins that are not efficiently presented as part of MHC complexes. Alternatively, HPV may have developed mechanisms that directly interfere with MHC expression and function as has been described for other DNA virus such as adenovirus, herpesvirus and pox viruses [62]. For example, interferon (IFN) stimulates expression of MHC class I and II as well as LMP2 and -7, which are part of the 20S proteasome degradation machinery used for the display of antigens to the cell surface [63–65]. Therefore, the inhibition of IFN sig-

naling by the HPV oncoproteins (see below) may be one of the mechanisms utilized by the virus to evade immune surveillance.

7. The efficacy of IFN treatment in HPV infection and associated disease

IFNs are a family of biological response modifiers that exhibit antiviral, antiproliferative and immunomodulating functions [66]. They consist of two different subtypes: type I IFNs (i.e. IFN-α/β) that are produced in virally infected cells, and type II IFN (i.e. IFN-γ) that is not virus inducible and is restricted to mitogen or cytokine-activated lymphoid cells such as T lymphocytes and natural killer (NK) cells [66]. In addition to the establishment of an antiviral state in uninfected cells, the elimination of virally infected cells is also critical in the host defense. In this context, type I IFNs exhibit a selective induction of apoptosis in virally infected cells and play a bifunctional role in limiting the spread of virus and eliciting an antiviral state in uninfected cells while promoting apoptosis in infected cells [67].

The treatment of HPV-infection and associated disease with IFNs has been under clinical experimentation. Theoretically, IFN treatment should result in the clearance of visible HPV lesions and elimination of the virus, even in cases of latent infections. However, despite the satisfactory results in some of the clinical studies, IFN treatment is still far from widespread therapy against HPV infection [68]. Studies on the effectiveness of IFN therapy in patients with CINs and cervical carcinomas have produced very inconsistent results and the conclusion regarding their efficacy has been controversial [69]. A compilation of data from various studies in vivo indicates that IFN-β is more effective than IFN-α and generally type II IFN (i.e. IFN-γ) more effective than type I (Table 1).

The molecular basis for this discrepancy in the efficacy of IFN treatment has not been determined, but it has been observed that expression of viral oncogenes, particularly E7, is significantly higher in the no responders than in responders [70]. Downregulation of HPV viral replication [70] and viral gene expression could be the reason for the positive clinical outcomes observed with IFNs [71–73]. However, studies with various cervical cancer cell lines in vitro showed that IFNs are unable to suppress HPV expression universally [69]. Thus, the efficacy of IFNs may be dependent upon the levels of expression of the HPV oncogenes, the distinct functions of the viral proteins in regulating IFN production and signaling, the complex interactions between the viral oncoproteins and cellular factors that affect both viral and host gene expression and function, and/or mutations unique to the infected cells that disrupt intracellular surveillance and regulatory mechanisms upon HPV infection [69].

8. IFN signaling and the Jak–Stat activation pathway

The mechanisms by which IFN transmit signals to the cell interior have been extensively studied in the recent years [74]. Type I IFNs (i.e. IFN-α/β) transduce their signals through the sequential activation of receptor associated *Janus* tyrosine kinases Jak1 and Tyk2 leading in tyrosine phosphorylation and activation of the signal transducers and activators of transcription Stat1 and Stat2 [74]. Activated Stat1/Stat2 heterodimers then translocate to the nucleus, where they associate with a 48 kDa DNA-binding protein [known as IFN stimulatory gene factor ISGF-3γ or IFN regulatory factor IRF-9 [75]] to form an active complex (known as ISGF-3) on the interferon response element (ISRE) [74] (Fig. 2). This element is known to mediate the induction of a number of functionally important IFN-stimulated genes (ISGs) including the translation initiation eIF-2α kinase PKR, 2′-5′ oligoA synthase, Mx, ISG-15 [74].

Type II IFN (i.e.IFN-γ) transduces its signals through the sequential activation of receptor associated Jak1/Jak2 and then Stat1. Activated Stat1 homodimers translocate to the nucleus and directly bind to members of the GAS (IFN-γ activated sequence) family of enhancers (Fig. 2). This element is known to mediate the induction of a distinct family of genes including IRF-1, IFP-53. For both types of IFN. Stat1 activation is mediated by a single phosphorylation site on tyrosine 701 [76]. In addition, phosphorylation of Stat1 on serine 727 is also required for maximal transcriptional activation [76]. Consistent with these observations, Stat1 null mice exhibit profound defects in IFN signaling, rendering them highly susceptible to infection with viruses and other pathogens [77,78].

9. Molecular actions of E6 and E7 in signaling induced by virus infection and IFNs

Several studies in vitro have demonstrated the ability of HPV oncoproteins to control signaling pathways that lead to the expression of IFNs and IFN-inducible genes. For example, it has been reported that HPV-16 E6 binds to the carboxyl-terminal domain of transcription factor IRF-3 and inactivates its transactivating function [79] (Fig. 3). IRF-3 is a member of the interferon regulatory factor (IRF) family whose members play a critical role in the regulation of the IFN α and β genes [80]. IRF-3 was originally characterized as a transcriptional factor that binds to ISRE. IRF-3 is part of a virus activated transcription factor complex includ-

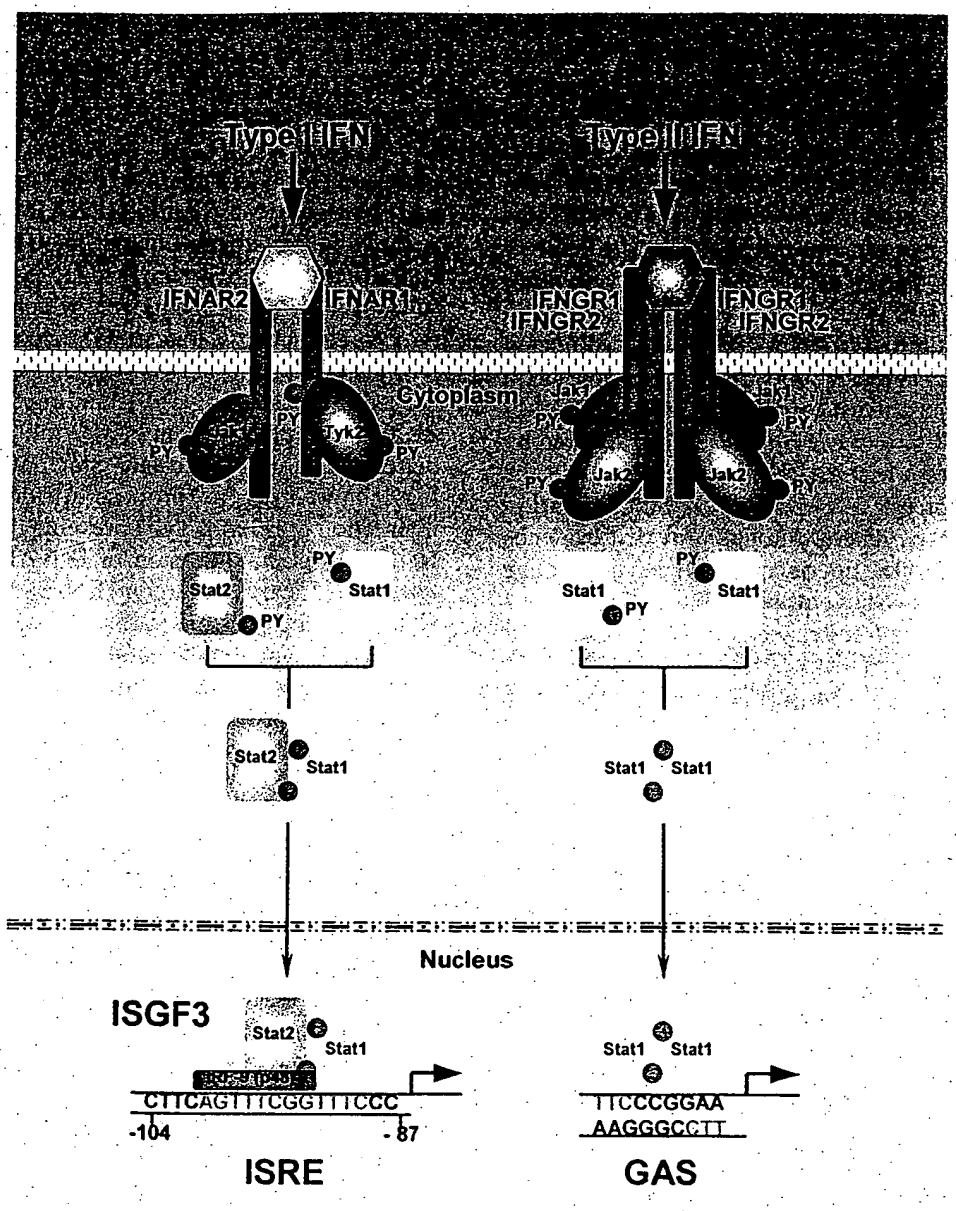

Fig. 2. A schematic model of activation of the Jak–Stat pathway by IFN: Binding of IFNs to their receptors results in the activation of the cytoplasmic tyrosine kinases of the *Janus* kinase (Jak) family, Jak1 and Tyk2 for IFN-α/β, and Jak1 and Jak2 for IFN-γ. Activated Jaks become autophosphorylated and subsequently phosphorylate the receptor on specific tyrosine residues, which function as docking sites for the *src* homology 2 (SH2) domain of Stat1 and Stat2. Tyrosine phosphorylation by Jaks results in heterodimerization of Stat1 and Stat2 or homodimerization of Stat1 and their nuclear translocation upon IFN-α/β or IFN-γ treatment, respectively. Stat1/Stat2 heterodimers bind to ISRE DNA sequence in the presence of a 48 kDa protein known as ISGF-3γ or IRF-9 to form the ISGF3 complex whereas Stat1 homodimers bind to GAS directly.

ing IRF-7, p300 and pCBP, whose activity is induced in response to viral infection [81]. Expression of HPV-16 E6 in human keratinocytes was able to diminish the induction of IFN-β gene expression by Sendai virus and consequently the expression of IFN-inducible genes [79]. Interestingly, inhibition of IRF-3-mediated transactivation was not observed with the benign HPV-6 E6 whereas the malignant HPV-18 E6 exhibited a modest binding to IRF-3 [79]. These finding indicated that inactivation of IRF-3 by E6 is probably specific for the HPV-16 type. Although IRF-3 is subjected to proteasome dependent proteolysis after virus infection this property is not modulated by E6 [79]. Considering the complex regulatory transcription pathways implicated in IFN-β gene transcription it is reasonable to speculate that E6 may interfere with other transcriptions factors involved in this process. Inasmuch as IRF-3 transactivation requires its interaction with CBP/p300 [81], inactivation of these co-activators by E6 [32,33] may also play a role in the inhibition of IFN-β gene transcription.

Another mechanism by which HPV infection impairs the antiviral and antiproliferative actions of IFN-α was reported by our group and involves the interaction of E6 with the tyrosine kinase Tyk2 [82]. We showed that expression of the high-risk HPV-18 E6 in human fibrosarcoma epithelial-like HT1080 cells results in inhibition of Jak–Stat activation in response to IFN-α. This inhibitory effect, however, was not shared by the low-risk HPV-11 E6. The DNA binding and transactivation capacities of ISGF3 were impaired by HPV-18 E6 expression after stimulation with IFN-α. This coincided with an impaired tyrosine phosphorylation of Tyk2, Stat2 and Stat1. In contrast to IFN-α, tyrosine phosphorylation and DNA binding of Stat1 were not affected by HPV-18 E6 in response to IFN-γ indicating that the inhibitory effect of HPV-18 E6 on Jak–Stat activation is specific for IFN-α. These findings provided evidence for a role of HPV-18 E6 oncoprotein as a negative regulator of the Jak–Stat pathway [82].

Further analysis of the molecular mechanisms that mediate the inhibition of Tyk2 activation by HPV-18 E6 led to the identification and partial characterization of

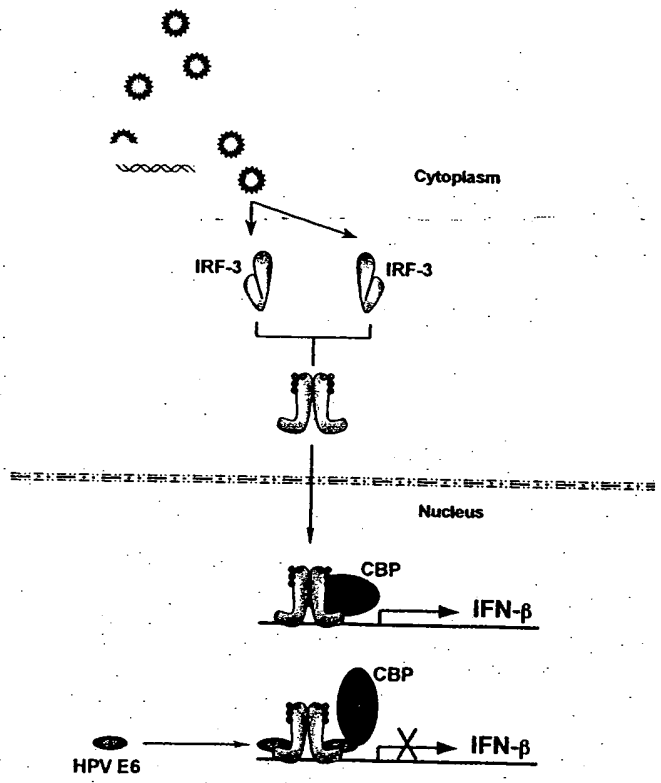

Fig. 3. Model of inhibition of IRF-3-mediated transactivation by 16-E6: IRF-3 exists in a latent state in the cytoplasm of infected cells. Virus infection leads to phosphorylation at a serine/threonine cluster within the C-terminus of the protein causing a conformational change in IRF-3 and leading to its nuclear translocation. Transcriptional activation of IFN-β promoter requires binding of IRF-3 to DNA and CBP as well as the presence of other transcriptional factors (not shown) that form the IFN-β promoter enhanceosome. The interaction of 16-E6 with IRF-3 [79] and CBP [32,33] may diminish the transactivation activities of the IRF-3/CBP complex thus decreasing IFN-β expression.

an interaction between E6 and Tyk2 [82]. Specifically, this interaction takes place preferably with both HPV-18 E6 and HPV-16 E6 and to a lesser extent with HPV-11 E6. Mapping of the interaction between the two proteins revealed that the JH_6-JH_7 domains of Tyk2, which are important for Tyk2 binding to the cytoplasmic portion of IFN-α receptor 1 chain (IFNAR1) [83,84], are also required for binding to E6 [82]. The JH_6 domain of Tyk2 contains a stretch of amino acids E–S–L–G very similar to the E–L–L/V–G sequence, which was described as an E6 interaction sequence [85]. Experiments are currently under way to determine the role of the E–S–L–G sequence in mediating Tyk2 binding to E6 and examine the function of E6-binding Tyk2 mutants in IFN-α-mediated activation of the Jak–Stat pathway. These findings allowed us to propose a model whereby the interaction of E6 with Tyk2 prevents the binding of Tyk2 to the cytoplasmic domain of IFNAR1 and the subsequent Tyk2 activation upon IFN-α stimulation (Fig. 4). Currently, the question rises as to whether Tyk2 inactivation is mediated by the ability of E6 to target proteins to proteasome dependent proteolysis. Although E6 does not affect the protein levels of Tyk2 in vitro (unpublished observations), specific degradation of phosphorylated (i.e. activated) form of Tyk2 in vivo is a possibility that is being examined.

The inactivation of Tyk2 by E6 is not the only mechanism utilized by the high-risk HPVs to block the Jak–Stat pathway. Interestingly, expression of HPV-16 E7 in the HPV-negative human epithelial cell line HaCaT was shown to inhibit the induction of IFN-α-inducible genes but had no effect on IFN-γ-inducible genes [86]. This inhibition correlated with the loss of the ISGF3 transcription complex as a result of inhibition of nuclear translocation of the IRF-9 (i.e. p48) component. This impaired nuclear translocation is most likely mediated by a direct interaction between E7 and IRF-9 (Fig. 5). Binding to IRF-9 requires the amino acids 17–37 of E7, a domain that includes the binding site to the Rb protein. Based on these data Barnard and McMillan [86] proposed that the ratio of E7 to IRF-9 may play a role in determining an effective response to IFN-α. That is, a patient with higher levels of IRF-9, or lower levels of E7, may be more likely to respond to IFN-α treatment. This notion is consistent with other observations that activation of ISGF3 is diminished in a number of HPV-positive cell lines [87]. Also, patients that do not respond to IFN-α treatment for HPV-positive condylomas have higher levels of E7 mRNA than those who are able to respond [70]. It would be of interest, however, to examine whether low-risk E7 also binds to IRF-9 with the same affinity as the high-risk protein, and whether the E7/IRF-9 interaction sufficiently accounts for the loss of ISGF3 formation in response to IFN-α.

The ability of E7 to interfere with IFN signaling was further demonstrated by its ability to bind to and inactivate the transcription factor IRF-1 [88,89]. IRF-1

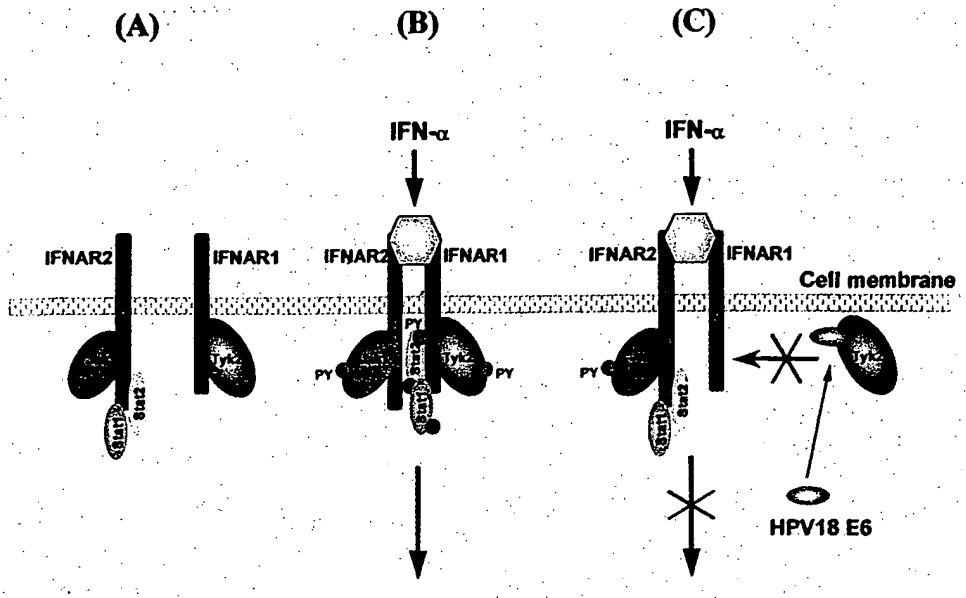

Fig. 4. Model of inhibition of Jak–Stat signaling by 18-E6: Binding of IFN-α to the receptor (A) leads to a cascade of tyrosine phosphorylation of Jak1, Tyk2, Stat2 and Sta1 molecules and (B) as explained in Fig. 2. The interaction of HPV-18 E6 with the JH_6-JH_7 domains of Tyk2 [82] may interfere with the association of Tyk2 with the cytoplasmic portion of the IFNAR1 thus preventing Tyk2 activation and phosphorylation of Stats.

was originally identified as an IFN-β promoter binding transcription factor and characterized as a critical mediator of IFN signaling induced by virus infection or IFN treatment [90]. Interestingly, IRF-1 overexpression inhibits cell growth and the introduction of activated c-Ha-ras oncogene alone is sufficient to transform embryo fibroblasts from IRF-1 knock out mice [90]. These data suggested that IRF-1 is a tumor suppressor gene product associated with the anti-proliferative effects of IFNs. Both HPV-16 and HPV-11 E7 proteins impair the transactivation activity of IRF-1 and this effect is mediated by a direct interaction between E7 and IRF-1 [88]. Binding of E7 to IRF-1 requires the Rb-binding portion of E7 and the carboxyl-terminal transactivation domain of IRF-1 [88]. The inhibition of IRF-1-dependent transactivation is most likely mediated by the recruitment of histone deacetylase (HDAC) by E7 to the IFN-β promoter (Fig. 6) [88]. The functional inactivation of IRF-1 by high and low risk E7 proteins could play an important role in the inhibition of IFN-β gene expression during viral infection. Inasmuch as both IRF-1 and p53 are required for the transcriptional activation of the cdk inhibitor p21 [91], their inactivation in HPV infected cells may represent major mechanisms that contribute into tumor formation and cervical carcinogenesis.

It is possible that HPVs has developed distinct mechanisms to block IFN action and the establishment of an antiviral state during early- or late-phase of infection. The results obtained in transformed cell lines with stable expression of E6 and/or E7 likely reflect conditions with prolonged or chronic viral infection and may not be accurate models of initial HPV infection. This notion has been supported by a recent study describing the global changes in gene expression in human keratinocytes latently infected with the high-risk HPV 31 using microarray analysis [92]. In this study, genes whose expression was repressed 2 fold or more by HPV proteins were listed into three groups. The first group consisted of regulators of cell growth such as p21, Mad, transgelin; the second group contained keratinocyte-specific genes including SprII, a small proline-rich protein found in UV-irradiated keratinocytes, and defensin; the third group consisted of IFN-inducible genes including Stat1 and 2′-5′ oligoA synthase [92]. Since Stat1 plays a crucial role in IFN-inducible gene transcription, the low basal level of its expression in HPV31 cells may account for the low level of various IFN-inducible genes and contribute to the impaired response to IFN signaling in HPV31 infected cells [92]. It would be important, however, to know which of the HPV-31 gene products are responsible for the transcriptional repression of Stat1, the mechanism of action, and whether Stat1 transcriptional repression is observed with low-risk HPVs.

10. Concluding remarks

HPVs have developed effective strategies to evade immune surveillance by downregulating both the production and action of IFNs. At the clinical level the disease frequently recurs when IFN therapy is discontinued, and the effect of IFNs is restricted to an anti-

proliferative rather than to an anti-viral or immunostimulatory action. Despite this unfavorable outcome, studies on the regulation of IFN pathways by HPV infection at the molecular level have provided and will continue to provide important insights into complex interactions between the viral oncoproteins and host factors that control innate immunity. The resulting knowledge will be useful in the design of novel strategies that combat HPV infection and associated disease. For example, a better understanding of the molecular mechanisms of inhibition of IRF-1, IRF-9 or Tyk2 by the HPV oncoproteins may lead to the development of therapies based on peptides that block the viral-host protein interactions and restore IFN signaling through endogenous control mechanisms. Another possibility is the development of anticancer therapies based on the ability of HPV infected cells to subvert IFN treatment. Significantly, the use of viruses with mild or arymptomatic infections in humans as antitumor agents is now under clinical experimentation [93–97]. Interestingly, it has been recently demonstrated that infection of various cancer cells with vesicular stomatitis virus leads to a selective killing of cells with a defective response to IFNs [98]. This may represent a new strategy for the treatment of IFN-non-responsive tumors. Whether this is a suitable treatment of HPV-associated tumorigenesis is an intriguing possibility that remains to be examined.

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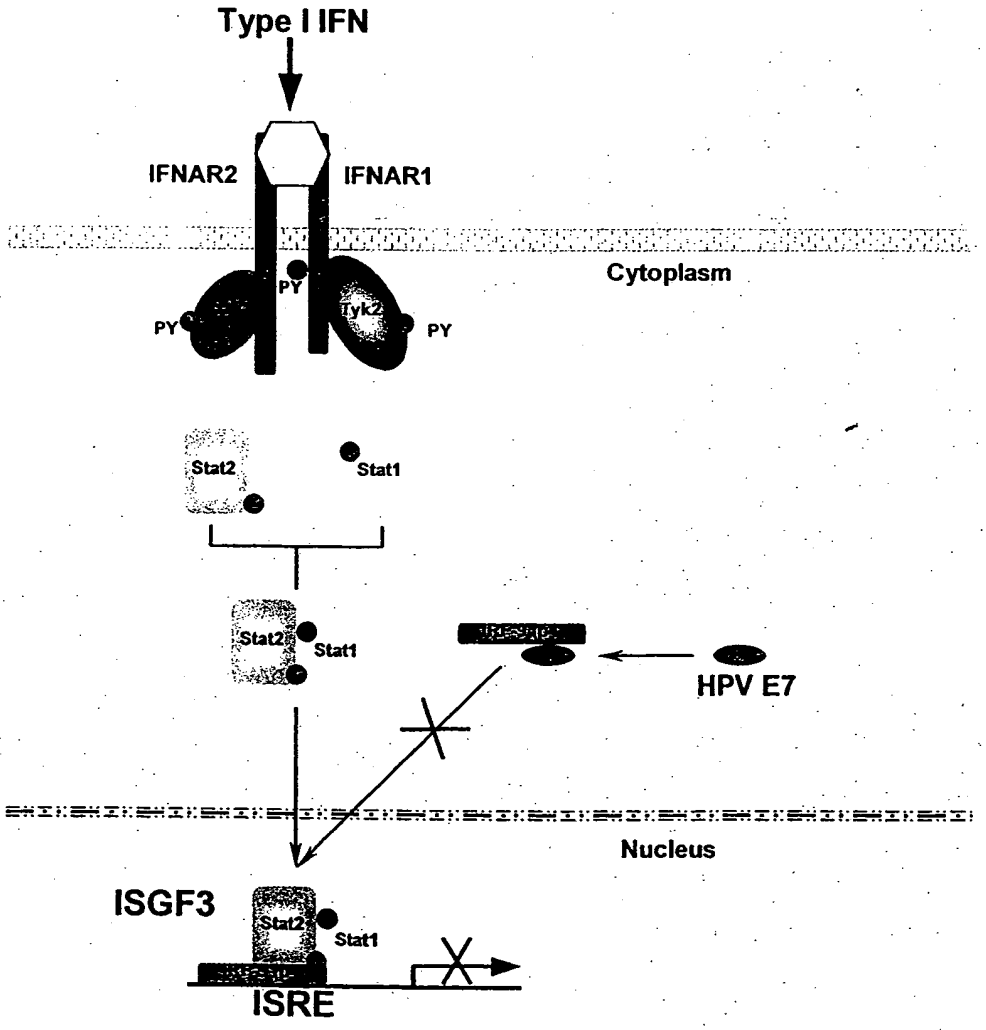

Fig. 5. Inhibition of IFN-α-induced ISGF3-dependent transcription by 16-E7: Transcriptional activation by IFN-α requires the formation of the ISGF3 complex, which consists of Stat1/Stat2 hetrodimers and IRF-9 (i.e. p48). HPV-16 E7 has been shown to bind to IRF-9 and inhibit its nuclear translation thus diminishing the DNA-binding and transactivation activities of ISGF3 [86].

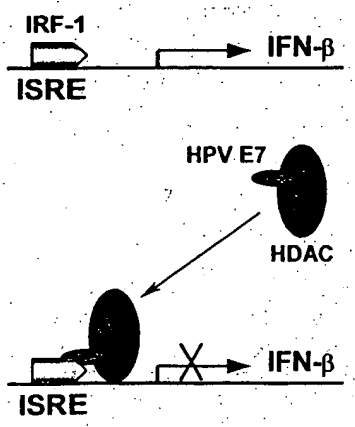

Fig. 6. Schematic model of IRF-1 inactivation by E7: Binding of IRF-1 to ISRE in the promoter of IFN-β and other IFN-inducible genes leads to transcriptional activation. HPV-E7 interacts with both IRF-1 and HDAC on the IFN-β gene promoter. Since HDAC mediates histone deacytelation of chromatin, such interactions were proposed to be responsible for the transcriptional repression of IRF-1 [88].

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Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: Implications for cervical carcinogenesis

SAEWHA JEON AND PAUL F. LAMBERT*

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, 1400 University Avenue, Madison, WI 53706

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ABSTRACT **In many cervical cancers, human papillomavirus type 16 (HPV-16) DNA genomes are found to be integrated into the host chromosome. In this study, we demonstrate that integration of HPV-16 DNA leads to increased steady-state levels of mRNAs encoding the viral oncogenes E6 and E7. This increase is shown to result, at least in part, from an increased stability of E6 and E7 mRNAs that arise specifically from those integrated viral genomes disrupted in the 3′ untranslated region of the viral early region. Further, we demonstrate that the A+U-rich element within this viral early 3′ untranslated region confers instability on a heterologous mRNA. We conclude that integration of HPV-16 DNA, as occurs in cervical cancers, can result in the increased expression of the viral E6 and E7 oncogenes through altered mRNA stability.**

Human papillomaviruses (HPVs) are small DNA viruses that infect epithelial cells. About 70 different genotypes have been identified to date, among which only a subset are associated with cervical cancers. More than 90% of cervical cancers contain these "high-risk" HPVs (HPV-16, -18, -31, and -33). In these cancers, two viral transforming genes, E6 and E7, are consistently expressed (1). The E6 and E7 proteins have been shown to interact with and inactivate the tumor-suppressor gene products p53 and pRb, respectively (34), and to immortalize human epithelial and fibroblastic cells as well as rodent fibroblasts (2–4). In transgenic mouse systems, expression of these genes leads to tumor formation (5–8). These studies have demonstrated the likely importance of E6 and E7 in cervical carcinogenesis.

The viral DNA genome of HPV-16 or -18 is often found integrated into the host chromosomes in cervical cancers (9–11). This viral DNA integration has been hypothesized to result in increased expression of E6 and E7 (12). To test this hypothesis, we have isolated a series of human cervical epithelial cell populations that harbor either extrachromosomal or integrated HPV-16 DNA (35). These cell populations were derived from a parental cell population, W12, that had been established from an HPV-16-positive cervical biopsy (13). Using these reagents, we have demonstrated that HPV-16 DNA integration correlates with increased expression of the viral E7 protein and with a selective growth advantage over cells harboring extrachromosomal HPV-16 DNA (35).

In the current study, we have sought to define a mechanism by which integration leads to increased expression of papillomaviral transforming genes. We demonstrate that the high levels of E7 protein seen in the integrated clones correlate with increased steady-state levels of E6- and E7-specific mRNAs, at least in part as a result of changes in their stability. This increased stability appears to be the result of the integrative disruption of the viral 3′ untranslated region (UTR) which we demonstrate contains an mRNA instability element.

MATERIALS AND METHODS

Southern and Northern Hybridization Analyses. Southern and Northern hybridizations were carried out by standard methods. For Southern analysis, a full-length HPV-16 DNA probe was used. For Northern analysis either an HPV-16 E6/E7-specific probe, generated by PCR using primers complementary to HPV-16 nt 79–101 and nt 883–864, or an HPV-16 3′ UTR-specific probe, generated by digestion of plasmid pHPV-16 with *Stu* I and *Xcm* I, which cleave HPV-16 DNA at nt 3871 and nt 4466, respectively.

S1 Nuclease Mapping Analysis. The E6/E7-specific cDNA fragment, used as an S1 probe, was generated by PCR amplification of an HPV-16-specific cDNA with primers complementary to HPV-16 nt 831–850 and 4152–4136 and subcloned into plasmid pGEM-3Z (Promega) to derive the clone pSJ314.71. The cDNA was first generated by reverse transcription of total cellular RNA from clone 20850e by using the primer complimentary to HPV-16 nt 4152–4136. Digestion of pSJ314.71 with *Nco* I and *Nar* I gave rise to a 1000-bp-long E6/E7 cDNA-specific probe (probe 1). The HPV-16 genomic DNA probe (probe 2) was generated by digestion of pHPV-16 with *Taq* I and *Nar* I, which cleave at HPV-16 nt 505 and 1309. The probes were ^{32}P-labeled at the 3′ end by nucleotide fill-in reactions. S1 nuclease analysis was performed as described (14).

Actinomycin D Assay. Total cellular RNA was extracted 0, 1, 2, 6, and 12 hr after administration of actinomycin D (5 μg/ml; Pharmacia). Northern analyses were performed with the E6/E7 probe described above.

***fos* Promoter Expression System.** NIH 3T3 mouse fibroblasts were cotransfected by the calcium phosphate precipitation method (15) with either pSJfosGlob or pSJfosGlob16 and pSVneo (16), which confers Geneticin (G418) resistance. Cells were placed under selection for G418 resistance for 2 weeks and colonies were pooled and expanded. The pooled population was starved for 25 hr in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% calf serum (completeness of synchronization was assessed by fluorescence-activated flow cytometry) and restimulated in DMEM with 10% calf serum. Digestion of pSJfosGLOB with *Hin*dIII and *Bam*HI gave rise to the β-globin-specific probe used for the Northern analyses. pSJfosGLOB was made by replacing the *Eco*RI–*Xcm* I fragment of pfos-βGLOB (human β-globin gene) (17) with that of pBBB3 (rabbit β-globin gene) (18), thus providing a unique *Bgl* II site in the β-globin 3′ UTR for cloning purposes. The 3′ UTR of HPV-16 was amplified by PCR using primers complementary to HPV-16 nt 4005–4025 and 4213–4195 and inserted into this unique *Bgl* II site of pSJfosGLOB, resulting in pSJfosGLOB16.

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Abbreviations: HPV, human papillomavirus; UTR, untranslated region; ARE, A+U-rich element.

*To whom reprint requests should be addressed.

RESULTS

To investigate the role that integration of HPV-16 DNA into human chromosome plays in cervical carcinogenesis, we have cloned and characterized cell populations harboring either extrachromosomal (e) or integrated (i) viral DNA (35) from the W12 cell population (13). A representative Southern analysis (Fig. 1) demonstrates that two of these clonal populations (20850e and 20863e) harbor ≈1000 copies of intact HPV-16 DNA, the majority of which were extrachromosomal as evidenced by the presence of the supercoiled form of HPV-16 DNA. Other clones exclusively harbor integrated viral DNA as judged by the absence of supercoiled DNA. Among these clones, two distinctly different patterns of integration were observed (Fig. 1); clones 20822 and 201402 gave rise to two junction-specific bands (type 1 integration), whereas clones 20831, 20862, and 20861 gave rise to both unique junction-derived fragments and unit-length 7.9-kb bands (type 2 integration).

To determine whether the heightened level of E7 protein expression in the integrated clones (35) was the consequence of increased levels of E7-specific mRNAs, we measured the steady-state level of HPV-16 E6/E7 mRNAs among the different clones by Northern analysis of total cellular RNA hybridized to an E6/E7-specific probe. A lower or similar level of E6/E7 mRNA expression was observed in the extrachromosomal clones compared with the integrated clones (Fig. 2). When corrected for differences in viral DNA copy number, a significantly higher level of E6/E7 mRNAs per viral genome copy accumulated in the integrated clones than in extrachromosomal clones (Table 1). When the same Northern blot was rehybridized to a radiolabeled probe specific for the 3′ UTR of the HPV-16 early region, the E6/E7 mRNAs in the extrachromosomal clones, but not in the integrated clones, were detected. The lack of hybridization of the 3′ UTR probe to E6/E7 mRNAs was unexpected in the case of the type 2

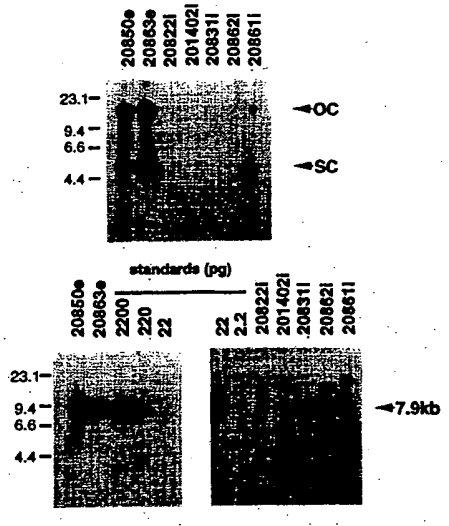

FIG. 1. State of HPV-16 DNA in the clonal populations derived from W12 cells. Southern analysis of both uncut sheared (*Upper*) and *Bam*HI-digested (*Lower*) total genomic DNA from clonal populations, hybridized with the full-length HPV16 probe. *Bam*HI cuts the HPV-16 genome once, at nt 6150. Because of the wide range of viral copy numbers among the cell populations, two different exposures of the blot of *Bam*HI-digested DNA are shown. Positions of open-circular (OC), supercoiled (SC), and 7.9-kb linearized HPV-16 DNA are indicated. Copy number of HPV-16 DNA was assessed in reference to the standards in which 2.2, 22, 220, and 2200 pg of cloned HPV-16 (corresponding to 1, 10, 100, and 1000 molecules of HPV-16 DNA per cell) were used in the reconstruction with DNA from HPV-negative Scc13ya cells.

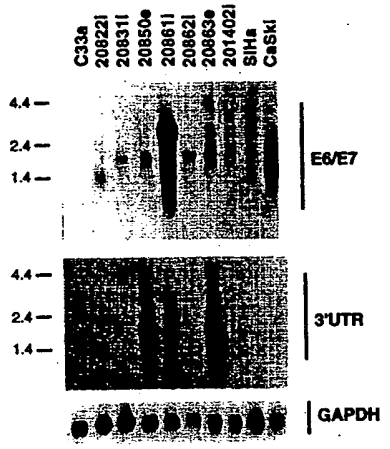

FIG. 2. Steady-state level of E6/E7 mRNA expression in the clonal populations. A Northern blot of total RNA from W12 clonal cell populations was sequentially hybridized with the probes specific to the HPV-16 E6/E7 region (*Top*), HPV-16 3′ UTR (*Middle*), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (*Bottom*) (provided to account for sample load variations on the gel). The C33a human cervical cancer cell line, which is HPV-negative, was used as a negative control. SiHa and CaSki are HPV-16-positive human cervical cancer cell lines. Note that the strong hybridization to the E6/E7-region probe was not completely removed in the 20661i lane when it was reprobed with the 3′ UTR probe. Additional experiments verified an absence of detectable hybridization to the 3′ UTR probe for RNA obtained from clone 20861i (data not shown).

integrated clones 20831, 20861, and 20862, since a significant fraction of the viral genomes present in these cells are unit length and therefore should give rise to E6/E7 mRNAs that possess the viral 3′ UTR. Interestingly, this result is consistent with what is observed in the cervical cancer cell line CaSki, which also has a type 2 integration event (19) (Fig. 2).

To more accurately characterize the structures of the E6/E7 mRNAs accumulating in our different clonal populations, we performed S1 nuclease mapping analysis using an E6/E7 cDNA probe (probe 1) (Fig. 3*A*). The 812-nt S1 product, indicative of the presence of E6/E7 mRNAs arising from intact viral genome (Fig. 3*A*), was detected only in the extrachromosomal clones (20850e and 20863e) (Fig. 3*B*). In contrast, S1 products of ≈380 nt were detected in the majority of integrated clones. These products were similar in size to that seen with CaSki RNA, suggesting that the integration events have led to the disruption of the viral sequences at nucleotide positions close to that in CaSki (20, 21). Consistent with this conclusion, we have cloned and sequenced the E6/E7 mRNAs arising from one of these clones, 20822i, and found the virus/cell junction to lie at HPV-16 nt 3732 (35). Two integrated clones, 20861i and 201402i, as well as the cervical cancer cell line SiHa, failed to give rise to any detectable S1 product with the E6/E7 cDNA probe, though one could detect E6/E7 mRNA with an E6/E7 genomic probe (probe 2) (Fig. 3*B*). The simplest interpretation of these results is that these E6/E7 RNAs utilize an alternative cell-specific 3′ splice site. With probe 2, two S1 products were obtained with clone 20861i RNA, and both were slightly larger than the product generated from RNAs utilizing the HPV-16 5′ splice signal at nt 880. This result suggests either that an alternative 5′ splice signal close to HPV-16 nt 880 is utilized in clone 20861i or that the viral/cellular junction is at this location.

The above described RNA analyses indicate that the E6/E7 mRNAs that accumulate in the clonal cell populations harboring integrated viral DNA arise predominantly from the copies of the viral genome disrupted by the integration event even in the type 2 integrated clones. This result led us to

Table 1. Properties of W12 clonal populations and cervical tumor cell lines

	Extrachromosomal	Integrated					
		Type 1			Type 2		
Cell population	20850/20863	20822	201402	SiHa	20831/20862	20861	CaSki
Viral copy number	1000	3	5	2	60	30	600
E6/E7 mRNA level*	0.2/0.4	0.2	0.3	0.3	0.2/0.3	2.3	1.0
	(0.12/0.24)	(40)	(36)	(94)	(2.0/3.0)	(48)	(1.0)
mRNA from intact HPV16†	+	–	–	–	–	–	–
E6/E7 mRNA half-life,‡ hr	3	6	6	ND	6	>12	6

Quantitation was performed by PhosphorImager analyses (Molecular Dynamics). Data on viral copy number was adopted from elsewhere (35). ND, not done.

*Obtained from data in Fig. 2 corrected to that of GAPDH mRNA. Values shown are normalized to E6/E7 mRNA level in CaSki. In parentheses, level of E6/E7 mRNA per viral genome copy relative to CaSki is indicated.

†Results from 3′ UTR-specific Northern blot (Fig. 2) and S1 nuclease mapping analysis (Fig. 3*B*) are summarized.

‡Calculated from actinomycin D mRNA-decay experiments (Fig. 4).

suspect that a possible cis effect was responsible for this selective accumulation of junction-derived E6/E7 mRNAs. The junction-derived E6/E7 mRNAs differ from the normal E6/E7 mRNAs in their 3′ ends. Given the well-documented role of 3′ UTRs in mRNA stability (22), we sought to determine whether the half-lives of E6/E7 mRNAs differed among the different clones. This was accomplished through the use of actinomycin D treatment, which blocks *de novo* RNA synthesis. The half-life of the E6/E7 mRNAs in the extrachromosomal clones 20850e and 20863e was 3 hr (Fig. 4). In comparison, the half-lives of E6/E7 mRNAs that accumulated in the integrated clones or cervical tumor cell lines were 6 to >12 hr. Thus, the actinomycin D experiments (summarized in Table 1) indicate that differences in mRNA stability might account in part for the selective accumulation of E6/E7 mRNAs from the junction copies of the viral genome.

Unstable mRNAs such as c-*fos* mRNA have been found to contain A+U-rich elements [AREs (18)] in their 3′ UTR that contribute to mRNA instability (23). A region that is 80% A+U (HPV-16 nt 4005–4213) is present within the 3′ UTR of E6/E7 mRNAs arising from intact viral copies. Importantly, the 3′ UTRs of E6/E7 mRNAs transcribed from integrated, disrupted HPV-16 genomes are replaced by cellular sequences with a lower A+U content: for example, 55% in CaSki (20) and 51% in 20822i (35). To see whether the ARE in the HPV-16 early-region 3′ UTR was sufficient to confer instability on an mRNA, the viral 3′ UTR was inserted into β-globin gene, a gene that expresses a highly stable mRNA, under the transcriptional control of the serum-responsive c-*fos* promoter (17, 18, 24, 25). This inducible promoter creates a short pulse of mRNA synthesis in synchronized populations of NIH 3T3 cells upon serum stimulation, providing a means to measure

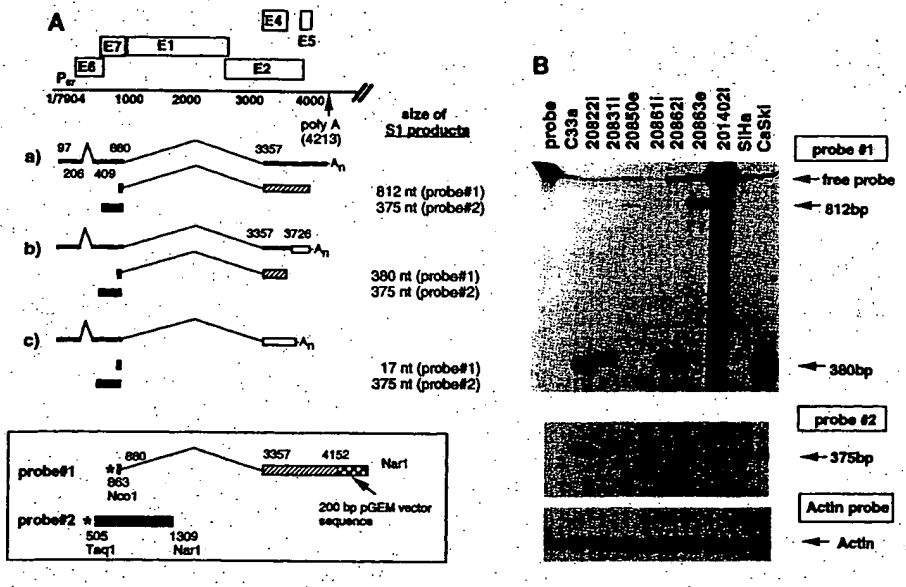

FIG. 3. E6/E7 mRNA expression in extrachromosomal clonal populations vs. integrated clonal populations and tumor cell lines. (*A*) Schematic illustration of potential E6/E7 mRNA species (solid boxes, HPV-16 RNA; open boxes, cellular sequences). Below each species are indicated S1 nuclease products protected by either the E6/E7 cDNA-derived probe (probe 1) or the HPV-16 genomic DNA-derived probe (probe 2) shown at the bottom (hatched and stippled boxes, HPV-16 DNA). (*a*) Representative HPV-16 E6/E7 mRNA species. The presence of the splice between HPV-16 nt 880 and 3357 and the viral 3′ UTR is common in all differentially spliced E6/E7 mRNAs from intact HPV-16 DNA. (*b*) The E6/E7 mRNA in CaSki cervical cancer cells. Splicing occurs between HPV-16 nt 880 and 3357 with fusion between viral and cellular sequences at nt 3726 (20). (*c*) The E6/E7 mRNA in SiHa cervical cancer cells. Splicing from HPV-16 nt 880 to a cellular 3′ splice signal has been detected (21). (*B*) S1 nuclease mapping analysis using either probe 1 (*Top*), probe 2 (*Middle*), or an actin probe (*Bottom*). The human actin probe was used as a control for sample load variation. Note that the S1 digestion of probe 1 in the 201402i sample was incomplete in the experiment presented. In other experiments, complete S1 digestion demonstrated the absence of any probe 1-specific S1 product for this RNA similar to that seen with SiHa and 20861i. Also note that levels of 20850i HPV-16-specific S1 products were very low (correlating with low levels seen by Northern analysis in Fig. 1); however, upon longer exposure the 375-bp probe 2 S1 product was detected.

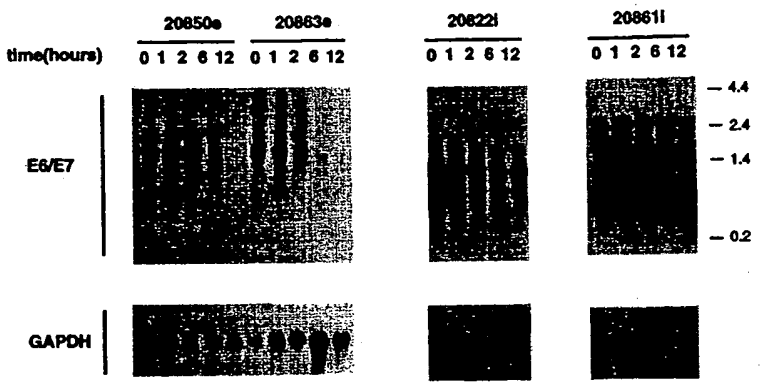

FIG. 4. Stability of E6/E7 mRNAs in extrachromosomal vs. integrated clonal populations. Northern hybridization to an E6/E7-specific probe was carried out with total cellular RNA obtained from clonal populations (20850e, 20863e, 20822i, and 20861i) 0, 1, 2, 6, and 12 hr after blocking of *de novo* transcription with actinomycin D. To determine half-lives as reported in Table 1, levels of E6/E7 mRNAs were corrected for variation in loading by comparison with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signal. The validity of the actinomycin D assay in our hands was determined by probing Northern blots with a c-*myc*-specific probe; the half-life of c-*myc* mRNA was reproducibly found to be 1 hr (data not shown). Actinomycin D experiments were performed four times and half-lives were reproducible (within 30%) among experiments.

the half-life of an mRNA. Using this approach, the ARE reduced the half-life of β-globin mRNA from >600 min to 90 min (Fig. 5). This reduction is similar to what has been observed with AREs from other short-lived mRNAs when assayed in the β-globin gene background (18, 19). Further, we found the E6/E7 mRNA, when expressed from the intact HPV-16 early region under the transcriptional control of the c-*fos* promoter, to have a half-life of only 20–40 min (data not shown). Based upon these findings, we conclude that the HPV-16 early-region 3′ UTR contains an mRNA instability element. Replacement of the viral 3′ UTR in E6/E7 mRNAs arising from integrative disruption of the HPV-16 early region would therefore be predicted to stabilize the viral mRNAs.

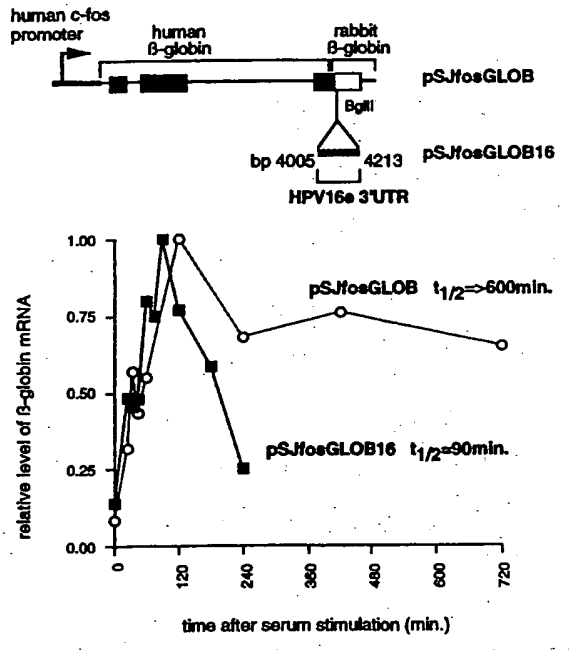

FIG. 5. Mapping the instability element present in the 3′ UTR of HPV-16 E6/E7 mRNA. (*Upper*) Schematic illustration of the plasmids used (hatched box, HPV16e 3′ UTR; stippled box, coding region of β-globin; open box, 3′ UTR of β-globin). (*Lower*) Levels of β-globin message in NIH 3T3 cells harboring either pSJfosGLOB or pSJfosGLOB16, plotted as a function of time after serum stimulation. The values were plotted relative to the peak levels of each specific RNA seen after serum stimulation. The time ($t_{1/2}$) at which the level of E6/E7 mRNA was half of the peak level is provided. Similar data were reproducibly obtained in two independent experiments.

DISCUSSION

In this report, we provide evidence that integration of HPV-16 DNA genomes in cervical epithelial cells leads to increased steady-state levels of viral mRNAs transcribed from the E6 and E7 oncogenes. Multiple mechanisms involving transcriptional derepression have been proposed previously to account for continued if not heightened expression of E6 and E7 oncogenes in cervical cancers (26, 27). We demonstrate that increased steady-state levels of E6/E7 mRNAs in cells harboring integrated viral DNA can result, at least in part, from an increase in mRNA stability through replacement of the HPV-16 early 3′ UTR, containing an mRNA instability element, with cellular sequences. This mechanism for dysregulated expression of papillomaviral oncogenes can be likened to that proposed to occur with the cellular protooncogenes c-*myc* and c-*myb* as a consequence of genomic rearrangements in cancers (28, 29). That HPV DNA integration leads to the removal of a virally encoded mRNA instability element provides: (*i*) a simple explanation for the selective accumulation of viral mRNAs arising from the integrated, disrupted copies of the viral genome in cancer cells in which most copies are integrated intact, such as is found in CaSki cells: (*ii*) a rationale for why integrative disruption of the viral genome as seen in cervical cancers occurs upstream of the papillomaviral early region 3′ UTR; and (*iii*) a potential explanation for why cellular sequences at the viral/cellular junction of an integrated copy of HPV-16 DNA in a particular cervical cancer were necessary for that HPV-16 DNA to confer efficient transformation of NIH 3T3 cells (30).

mRNA half-life is thought to be regulated through cis elements contained within the mRNAs. One such element controlling RNA stability is a specific sequence motif of the consensus sequence 5′-UAUUUAU-3′ present in multiple copies within the A+U-rich 3′ UTR of short-lived mRNAs (22). The ARE containing these sequence motifs within the HPV-16 early 3′ UTR sequences was found to be sufficient to confer instability on the β-globin mRNA (Fig. 5). Other levels of posttranscriptional regulation are also known to occur. For example, the 3′ UTR of the late genes of papillomiviruses has been implicated in modulating the steady-state levels of a distinct set of viral mRNAs that encode the viral structural (capsid) proteins (31, 32). Unlike the early 3′ UTR which we

have demonstrated to function at the level of mRNA stability, the late-region 3′ UTR has been found to modulate the steady-state levels of late mRNAs at the level of mRNA processing or nuclear transport (33). Thus, papillomaviruses use multiple posttranscriptional mechanisms for modulating expression of their genes.

We found the HPV-16 early 3′ UTR to confer a >7-fold reduction in β-globin mRNA half-life. Correspondingly, we found a 2- to >4-fold increase in half-life for E6/E7 mRNAs arising from viral genomes disrupted by integration. Yet the differences in the relative abundance of E6/E7 mRNAs per viral genome copy in the integrated versus extrachromosomal clones ranged from 4- to 400-fold. Thus, it is likely that the additional levels of control of gene expression must be affected by integration. The hypermethylated state of HPV-16 DNA in the W12 integrated clones compared with that in the extrachromosomal clones (36) indicates that transcriptional activity of the integrated viral genomes is not heightened. Nevertheless, it has been proposed (27) that viral DNA integration, as found in cervical cancers, might cause derepression of E6/E7 mRNA synthesis, through the integrative disruption of the viral E2 gene, which encodes a transcriptional repressor of the E6/E7-specific P_{97} promoter. Since the integration events in our W12 clonal populations also result in the disruption of the E2 gene, derepression of the P_{97} promoter may contribute to the increased steady-state levels of E6/E7 mRNAs in our integrated clones in addition to the altered mRNA stability. An alternative mechanism of transcriptional derepression has been recently identified, in which deletion of binding sites for the cellular transcription factor, YY1, located upstream of the P_{97} promoter results in loss of YY1-mediated repression of E6/E7 mRNA synthesis (26). We failed to detect any deletions within the HPV-16 genomes present in our clones that would be indicative of the disruption of putative YY1 sites upstream of the P_{97} promoter. It is perhaps significant that the majority of independent integration events that we have characterized in the W12 cell clones result in viral/cellular junctions that are clustered in a short region of the viral genome >300 nt upstream of the 3′ UTR. It is not known whether this region contains cis elements that affect steady-state levels of E6/E7 mRNAs independently of or in concert with the 3′ UTR.

The W12 parental cell population was a gift from Dr. Margaret Stanley. We thank Drs. David Herrick and Jeff Ross for advice on mRNA stability and Denis Lee for assistance with tissue culture. We thank Walter Hubert and Drs. Anne Griep, Jeff Ross, and Bill Sugden for critical review of the manuscript. This work was supported by Public Health Service Grants CA22443 and CA07175 and by American Cancer Society Grant JFRA-393.

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Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways

Edward C. Goodwin and Daniel DiMaio*

Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

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Most cervical carcinomas express high-risk human papillomaviruses (HPVs) E6 and E7 proteins, which neutralize cellular tumor suppressor function. To determine the consequences of removing the E6 and E7 proteins from cervical cancer cells, we infected HeLa cells, a cervical carcinoma cell line that contains HPV18 DNA, with a recombinant virus that expresses the bovine papillomavirus E2 protein. Expression of the E2 protein resulted in rapid repression of HPV E6 and E7 expression, followed ~12 h later by profound inhibition of cellular DNA synthesis. Shortly after E6/E7 repression, there was dramatic posttranscriptional induction of p53. Two p53-responsive genes, mdm2 and p21, were induced with slightly slower kinetics than p53 and appeared to be functional, as assessed by inhibition of cyclin-dependent kinase activity and p53 destabilization. There was also dramatic posttranscriptional induction of $p105^{Rb}$ and p107 after E6/E7 repression, followed shortly thereafter by induction of p130. By 24 h after infection, only hypophosphorylated $p105^{Rb}$ was detectable and transcription of several Rb/E2F-regulated genes was dramatically repressed. Constitutive expression of the HPV16 E6/E7 genes alleviated E2-induced growth inhibition and impaired activation of the Rb pathway and repression of E2F-responsive genes. This dynamic response strongly suggests that the p53 and Rb tumor suppressor pathways are intact in HeLa cells and that repression of HPV E6 and E7 mobilizes these pathways in an orderly fashion to deliver growth inhibitory signals to the cells. Strikingly, the major alterations in the cell cycle machinery underlying cervical carcinogenesis can be reversed by repression of the endogenous HPV oncogenes.

High-risk human papillomaviruses (HPVs) such as HPV18 play a central role in the development of essentially all cases of cervical carcinoma (1). However, carcinoma develops infrequently even after infection by these HPV types, and it typically occurs years to decades after the initial infection. Two HPV oncogenes, E6 and E7, are expressed in cervical carcinomas and carcinoma-derived cell lines. The E6 and E7 proteins can immortalize cultured primary human keratinocytes, but these immortalized cells are not tumorigenic unless additional, undefined genetic events occur. These observations imply that the viral oncogenes do not directly induce tumor formation but rather set in motion a series of events that may ultimately result in tumorigenicity.

The high-risk HPV E6 and E7 proteins exert profound effects on the tumor suppressor proteins p53 and $p105^{Rb}$ (1). These tumor suppressor proteins normally control signaling pathways that regulate the cell cycle and monitor and protect the integrity of the genome. p53 is a transcription factor that activates transcription of a variety of genes including $p21^{Waf1/CIP1/SDI1}$ (p21) (reviewed in ref. 2). p21 directly inhibits the activity of cyclin-dependent kinase (cdk) complexes, which are required for cell cycle progression. Transcription of the mdm2 gene is also induced by p53. mdm2 in turn binds to p53 and stimulates its degradation in a negative feedback loop that controls p53 levels. $p105^{Rb}$ and the retinoblastoma (Rb) family members p107 and p130 regulate the activity of E2F transcription factors, which control transcription of a variety of genes required for cell cycle progression (reviewed in refs. 3 and 4). Hypophosphorylated Rb family members bind to E2F family members, thereby forming complexes that actively repress transcription of cell cycle genes (5–13). Phosphorylation of Rb proteins by cdks disrupts these complexes. Disruption of these complexes impairs repression and increases the concentration of unbound E2F family members, some of which, like E2F1, can stimulate transcription (3, 4).

The high-risk HPV E6 protein binds to p53 and targets it for accelerated ubiquitin-mediated degradation, and the high-risk HPV E7 protein binds to hypophosphorylated members of the retinoblastoma family, resulting in their destabilization and the disruption of Rb/E2F repressor complexes (14–21). Therefore, levels of p53 and hypophosphorylated Rb are typically low in cells expressing the E6 and E7 proteins. As a consequence of these interactions, expression of high-risk HPV E6 and E7 proteins in cultured cells disrupts cell cycle checkpoint control and results in increased rates of mutagenesis and genetic instability (18, 22–27). Thus, expression of the E6 and E7 proteins may facilitate acquisition of the additional genetic changes that drive carcinogenic progression. Similar processes appear to occur during cervical carcinogenesis *in vivo*. For example, HeLa cells, an aneuploid tumorigenic cell line derived from a malignant human cervical carcinoma, express E6 and E7 proteins from integrated HPV18 DNA and display aberrant checkpoint control (28, 29).

Most cervical carcinomas and cervical carcinoma cell lines, including HeLa cells, harbor wild-type p53 and $p105^{Rb}$ genes (30, 31). Thus, the growth regulatory machinery active in normal cells may be intact in these carcinoma cells but masked by expression of the HPV E6 and E7 proteins. To analyze the consequences of removing the HPV E6 and E7 proteins from cervical carcinoma cells, we and others have exploited the ability of the bovine papillomavirus (BPV) and HPV E2 proteins to repress E6/E7 transcription by binding directly to the HPV early promoter (32–41). Introduction of an ectopic E2 gene into a number of cervical carcinoma cells lines results in a great reduction in E6/E7 mRNA and in substantial growth inhibition (32–36, 38, 42). E2-mediated growth inhibition is observed only in cells containing HPV DNA, and E2-induced reduction in HeLa cell colony formation is prevented by constitutive expression of the HPV16 E6 and E7 genes (32, 38, 41, 42), indicting that repression of E6/E7 is required for the growth inhibitory effect.

BIOCHEMISTRY

Abbreviations: HPV, human papillomavirus; Rb, retinoblastoma; cdk, cyclin-dependent kinase; BPV, bovine papillomavirus.

*To whom reprint requests should be addressed. E-mail: daniel.dimaio@yale.edu.

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We used a recombinant SV40-based viral vector to express the E2 protein in cervical carcinoma cells (32). Two days after infection with this virus (but not after infection with viruses expressing inactive mutant E2 proteins), HeLa cells display profound growth inhibition, accumulating with a G_1/G_0 DNA content (32, 34, 42). At this time, HPV18 E6/E7 expression is repressed and p53, p21 and hypophosphorylated $p105^{Rb}$ are induced. However, these experiments examined a single time point after the imposition of severe growth inhibition and therefore provided a static snapshot of the cellular response to E2 expression. The sequence of biochemical events that occurs after E6/E7 repression is not known, nor is it known whether the cells arrested because of the orderly reestablishment of normal growth control mechanisms or to catastrophic derangement of cellular metabolism. Here, we examined the sequence of biochemical events induced by expression of the E2 protein in cervical carcinoma cells. This kinetic analysis revealed a dynamic and complex sequence of activating and repressing events in cell cycle regulatory components that strongly implied that the p53 and Rb tumor suppressor signaling pathways are intact in HeLa cells and able to transduce a growth inhibitory signal once HPV oncogene expression is extinguished. These findings have important implications for the pathogenesis and treatment of cervical carcinoma.

Materials and Methods

Cells and Virus Preparation. HeLa cells were maintained in standard media as described (42). The pPava-5'BΔS viral vector (42), which contains a wild-type BPV E2 gene but no SV40 T antigen gene and a disrupted BPV E5 gene, was further modified by replacing the AUG start codon for the internally initiated, E2 trans-repressor protein with an ATC codon, a mutation with no apparent effect on the E2 activities measured here (32). This repressor minus construct was renamed pPava-5'BΔS-RMC. Viral stocks were prepared and titered as described previously (38), and mock-infected cells were used as controls. Cellular DNA synthesis assays were performed in quadruplicate as described (42), with the modification that infections were at a multiplicity of infection of 20 and that [^{3}H]thymidine labeling was performed for only 2 h. Recombinant retroviruses expressing HPV16 E6/E7 and control retroviruses were obtained from Denise Galloway (Fred Hutchinson Cancer Research Institute) (43). After infection with these viruses and selection for G418-resistance, individual clones of drug-resistant HeLa cells were expanded into cell lines for analysis.

RNA Analysis. Cells were infected as described previously (42), and cell pellets were harvested and frozen at −80°C until fractionation. Total cellular RNA was purified by using Trizol reagent (Life Technologies), and 5 μg of RNA was subjected to formaldehyde-agarose gel electrophoresis, transferred to Nytran (Schleicher & Schuell) and crosslinked to the membrane by UV irradiation. The immobilized RNA was hybridized with the indicated random prime-labeled cDNA, and the signal was detected and quantified with a PhosphorImager (Molecular Dynamics). Sequential hybridizations were performed after stripping the previous probe from the membrane. RNA levels were normalized to the signal obtained with ubiquitin mRNA.

Immunoblotting and cdk2 Kinase Activity. Protein for immunoblotting was prepared from the Trizol extracts after isolation of the RNA as described (38). Five micrograms of extracted protein was resolved by denaturing PAGE, transferred to an Immobilon-P membrane and probed with the antibodies specific for the following proteins: E2F1 (catalogue no. 05-379) from Upstate Biotechnology (Lake Placid, NY); p107 (sc-318), p130 (sc-317), mdm2 (sc-965), cdc25A (sc-7389), cdk2 (sc-748), and p21 (sc-397), all from Santa Cruz Biotechnology; p53 (15801A) and p105Rb (14001A), both from PharMingen; and cyclin A (from H. Zhang, Yale University). To measure cdk activity, HeLa extracts were immunoprecipitated as previously described (38), and histone H1 kinase activity was determined and quantitated with a PhosphorImager. After subtraction of the small signal resulting from kinase reactions after immunoprecipitation with nonimmune, species-matched antibodies, the signals were normalized to mock-infected controls.

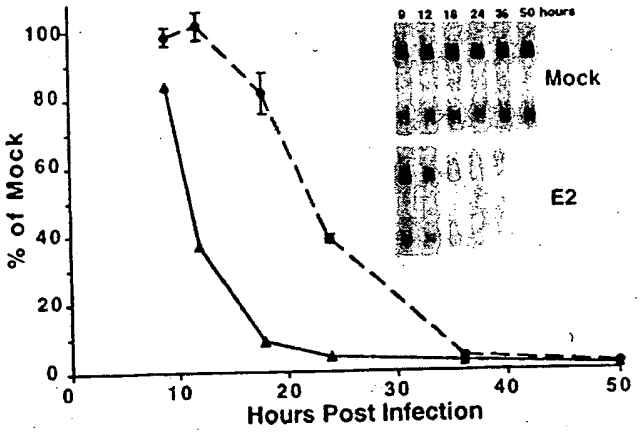

Fig. 1. Time course of HPV E6/E7 repression and growth inhibition. HeLa cells were infected or mock-infected, and, at the indicated time after infection, RNA was analyzed for HPV E6/E7 expression by Northern blotting (solid line), and cellular DNA synthesis was determined by incorporation of tritiated thymidine (dashed line). The error bars indicate two standard deviations of the mean. (*Inset*) Repression of HPV E6/E7 expression. Northern blot described above. RNA was isolated at the indicated hours after E2 infection (*Lower*) or mock-infection (*Upper*), electrophoresed, transferred, and probed with a radiolabeled HPV18 E6/E7 DNA fragment. The signal obtained was quantitated, normalized for the signal obtained with a ubiquitin probe, and expressed as the percentage of the normalized signal obtained with RNA from mock-infected cells.

Results and Discussion

E2-Mediated Inhibition of DNA Synthesis and Repression of HPV Gene Expression. We used a recombinant BPV/SV40 virus to introduce the BPV E2 gene into HeLa cells to determine the timing of events after expression of the full-length E2 protein. To measure cellular DNA synthesis, the cells were subjected to a 2-h pulse of [^{3}H]thymidine at various times after infection with the E2 virus at a multiplicity of 20 infectious units per cell or after mock infection, and acid-insoluble radioactivity was determined. As shown in Fig. 1, there was no difference in thymidine incorporation between infected and control cells at 12 h after infection. By 18 h after infection, infected cells showed a modest inhibition of DNA synthesis compared with mock-infected cells. The extent of inhibition increased with time, with DNA synthesis being approximately 40% of control levels by 24 h after infection and less than 5% by 36 h. These results demonstrated that the E2 protein exerted profound biological effects in the vast majority of cells in the population and established the time frame against which to measure biochemical changes in these cells.

Northern blotting was used to analyze expression of HPV18 E6/E7 mRNA at various times after infection or after mock-infection (Fig. 1 *Inset*). By 9 h after infection with the E2 virus, there was a reduction in HPV E6/E7 expression in infected cells compared with control cells. Quantitation in comparison to ubiquitin mRNA revealed that the level of E6/E7 RNA in E2-expressing cells was less than 40% the level in control cells by 12 h after infection and less than 10% by 18 h (Fig. 1). Expression of the E7 protein was repressed with similar kinetics

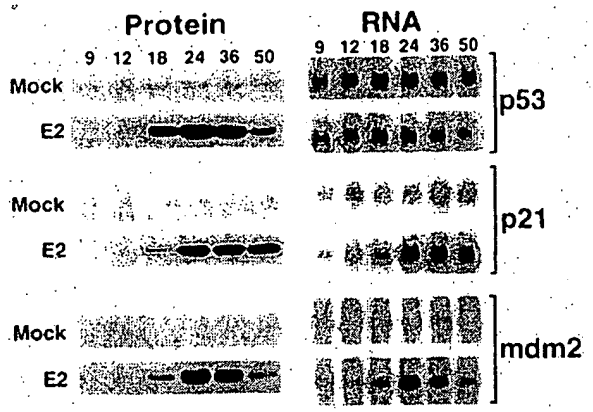

Fig. 2. (*Left*) Western analysis of p53 pathway. HeLa cell proteins were harvested at the indicated hours after E2 infection or mock-infection, electrophoresed, transferred, and probed with antibodies specific for p53, p21, or human mdm2, as indicated. (*Right*) Northern analysis of p53 pathway. HeLa cell RNA was isolated at the indicated hours after E2 infection or mock-infection. After electrophoresis and transfer, p53, p21, and mdm2 mRNA was detected by hybridization to the appropriate radiolabeled cDNA probe.

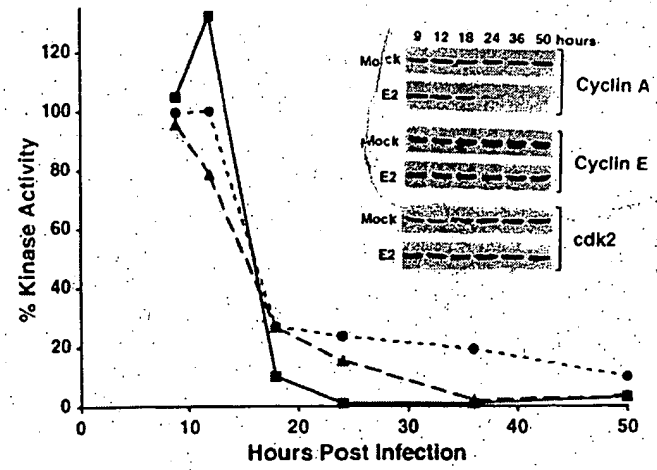

Fig. 3. Cyclin-dependent kinase activity. HeLa cell extracts were prepared at the indicated hours after E2 infection or mock-infection. After immunoprecipitation with control antibodies or antibodies specific for cyclin E (dotted line), cdk2 (dashed line), or cyclin A (solid line), kinase activity toward histone H1 was measured. The signal obtained was quantitated and expressed as the percentage of the corrected signal obtained with mock-infected samples. (*Inset*) Western analysis of cdk components. HeLa cell proteins were harvested at the indicated times from mock-infected or E2-infected cells. After electrophoresis and transfer, samples were probed with antibodies specific for cyclin A, cyclin E, and cdk2, as indicated.

(see Fig. 4). Therefore, HPV repression was a relatively early event after infection of HeLa cells with an E2-expressing virus and in fact was the earliest biochemical change we have detected. Importantly, HPV E6/E7 repression clearly preceded inhibition of DNA synthesis by about 12 h, a result consistent with repression playing a causal role in growth arrest.

Activation of the p53 Tumor Suppressor Pathway. The stability of p53 is increased in growth-arrested HeLa cells (32), an effect presumably due to the loss of the HPV18 E6 protein, which otherwise promotes accelerated, ubiquitin-mediated degradation of p53. Furthermore, in these growth-arrested cells, expression of the p53-responsive p21 gene is induced at the transcriptional level. Here, we used immunoblotting to determine the steady state level of p53 and two of its transcriptional targets, p21 and mdm2, at various times after infection (Fig. 2 *Left*). There was little change in the abundance of these proteins in mock-infected cells. In response to E2 expression, p53 displayed a complex kinetic profile, showing a dramatic induction by 18 h, followed by a drop after 24 h, so that by 50 h after infection the steady state level of p53 was only modestly higher than that observed in mock-infected cells. The mdm2 protein also showed a complex profile, with maximum levels attained at 24 h, after which there was a significant decline from the peak levels. p21 was induced with kinetics similar to mdm2, but its expression persisted for at least 50 h. p53 induction was approximately half-maximal by 18 h, whereas mdm2 and p21 induction was much less pronounced at this time point.

Because p53 and mdm2 displayed a similar expression pattern, namely an initial induction by the E2 protein followed by a decline, it seemed likely that these changes in the levels of the mdm2 protein were due to p53-mediated regulation of mdm2 transcription. Northern blotting demonstrated that mdm2 RNA levels did in fact rise and fall in parallel with mdm2 protein levels (Fig. 2 *Right*). Similarly, p21 was induced at the mRNA level. Consistent with the model that p21 and mdm2 induction was mediated by p53-transcriptional activation, neither gene was induced by E2 expression in HT-3 cells, an HPV30-containing cervical carcinoma cell line that expresses a transactivation-defective p53 protein (refs. 30, 31, and 38; data not shown). If the loss of HPV E6-directed degradation of p53 is responsible for the initial increase in p53 levels in HeLa cells, and the imposition of mdm2-directed degradation is responsible for the later decline, then the level of p53 mRNA should not change after expression of the E2 protein. In accord with this prediction, p53 mRNA levels changed little during the course of infection (Fig. 2 *Right*), despite the dramatic fluctuations in the level of p53 itself.

Because p21-mediated inhibition of cdk activity is an important consequence of p53 activation, we measured the kinase activity of cdk complexes *in vitro*. HeLa cell extracts prepared at various times after infection were immunoprecipitated with control antibodies or antibodies recognizing cdk2, cyclin A, and cyclin E, and the kinase activity of the immunoprecipitates toward histone H1 was measured. As shown in Fig. 3, E2 expression reduced the activity of all three types of cdk complexes, compared with complexes isolated from mock-infected cells. Inhibition of kinase activity was substantial by 18 h after infection and persisted for the duration of the experiment. To determine whether the components of cdk complexes were expressed at a reduced level in HeLa cells, we used immunoblotting to measure the expression of cdk2, cyclin A, and cyclin E. As shown in Fig. 3 *Inset*, the levels of cdk2 and cyclin E in cells expressing the E2 protein did not differ from those in mock-infected cells at any time during the course of the experiment. In contrast, cyclin A levels were essentially unchanged during the first 18 h after E2 infection and then declined to undetectable levels by 36 h after infection.

Our results suggest that the following sequence of events occurred in the p53 pathway. Binding of the E2 protein to the HPV18 early promoter caused transcriptional repression of E6/E7 expression. The resulting decay in the E6 protein reduced the amount of p53 targeted to the ubiquitin degradation system, leading to increased levels of p53. p53 induction caused increased transcription of the p21 and mdm2 genes and the accumulation of p21 and mdm2 proteins. As the mdm2 protein accumulated, it targeted p53 for accelerated degradation, leading to a posttranscriptional drop in p53 levels. As p53 levels dropped, it no longer induced mdm2 transcription, and levels of mdm2 RNA and protein dropped. This kinetic analysis provided evidence that the p53/mdm2 negative feedback loop is intact in

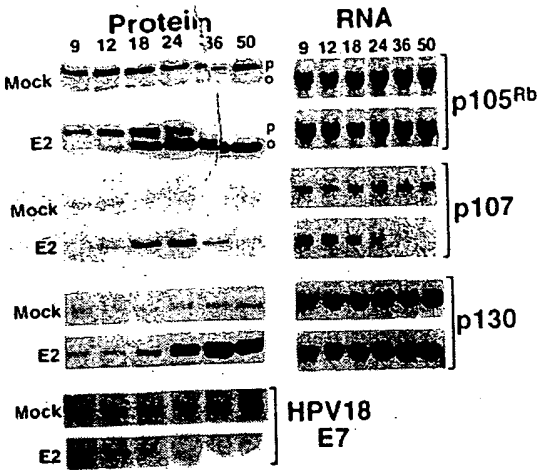

Fig. 4. (*Left*) Western analysis of retinoblastoma family members. HeLa cell protein was prepared at the indicated hours after E2 infection or mock-infection. After electrophoresis and transfer, specific antibodies were used to detect HPV18 E7, $p105^{Rb}$, p107, and p130. The hyperphosphorylated (p) and hypophosphorylated (o) form of $p105^{Rb}$ are indicated. (*Right*) Northern analysis of retinoblastoma family members. HeLa cell RNA was prepared at the indicated hours after mock-infection or E2 infection. After electrophoresis and transfer, $p105^{Rb}$, p107, and p130 mRNA were detected by hybridization to the appropriate radiolabeled cDNA probe.

HeLa cells. p21 mRNA levels also dropped at times later than the 50-h time point analyzed here (data not shown). The induced p21 bound to cdks and inhibited their activities, an effect that was reinforced by the absence of cyclin A in the case of total cdk2 and cyclin A-associated cdk activity.

Activation of the Retinoblastoma Tumor Suppressor Pathway. We previously reported that there is a marked increase in the levels of the hypophosphorylated form of $p105^{Rb}$ in E2-arrested cervical carcinoma cells, presumably due to reduction in proteosome-mediated degradation, as well as a reduction in the amount of the hyperphosphorylated form (34, 38, 44). Here, as shown in Fig. 4 *Left*, we used immunoblotting to examine the E7 protein and the Rb family members $p105^{Rb}$, p107 and p130 at various times after infection. A reduction in E7 protein expression was clearly evident by 12 h after infection, in parallel with the decrease in HPV E6/E7 RNA level. By 18 h after infection, there was an abrupt and dramatic induction of the level of hypophosphorylated $p105^{Rb}$ that increased until 24 h and persisted throughout the course of the experiment. In addition, at later times, hyperphosphorylated $p105^{Rb}$ disappeared, an effect likely due to the decline in cdk activity. Induction of hypophosphorylated $p105^{Rb}$ before the reduction in the hyperphosphorylated form was observed in multiple independent experiments. p107 was induced with similar kinetics as that observed for hypophosphorylated $p105^{Rb}$, but at later times the level of p107 dropped to that found in proliferating HeLa cells. p130 was induced more gradually than $p105^{Rb}$ or p107, with a significant increase only evident by 24 h, and the level of p130 remained elevated. The reciprocal expression of p107 and p130 correlates with a shift from cellular proliferation to a nonproliferative state in other systems as well (e.g., see citations in ref. 44).

If the increase in the abundance of the Rb proteins as infection proceeded was due to posttranslational stabilization as a consequence of the disappearance of the E7 protein, then the amounts of Rb family member mRNA are predicted not to increase. In support of this model, there was little increase in $p105^{Rb}$, p107, or p130 mRNA at any time after E2 expression (Fig. 4 *Right*). In contrast, the decline in p107 levels at later

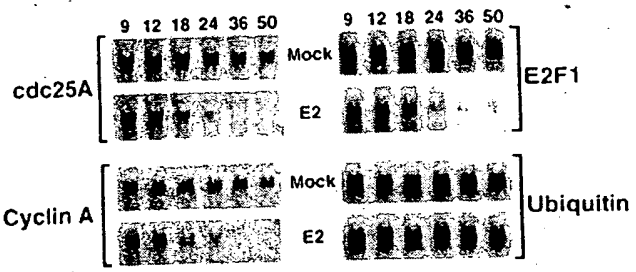

Fig. 5. Northern analysis of E2F-responsive genes. HeLa cell RNA was prepared at the indicated hours after mock-infection or E2 infection. After electrophoresis and transfer, E2F1, cyclin A, cdc25A, and ubiquitin mRNA were detected by hybridization to the appropriate radiolabeled cDNA probe.

times was due to a reduction in the amount of p107 mRNA as infection proceeded.

Rb family members exert their effects in large part by regulating the activity of E2F transcription factors, which themselves regulate the expression of genes involved in cell cycle progression. Here, we examined the time course of mRNA expression for four E2F-regulated genes, p107, cyclin A, E2F1, and cdc25A (3, 4, 45). In response to E2 expression, the level of these four mRNAs underwent a dramatic reduction, first evident at 18 h after infection with the E2 virus [Fig. 4 (p107) and Fig. 5], and the corresponding proteins underwent a similar reduction in amount [Fig. 3 *Inset* (cyclin A), and Fig. 4 (p107); data not shown for cdc25A and E2F1]. The repression of these E2F responsive genes in the same time frame as the induction of hypophosphorylated $p105^{Rb}$ strongly suggest that repression was due to a common, Rb-mediated mechanism. Elsewhere, we show that E2-induced repression of cdc25A expression in cervical carcinoma cells was mediated by increased formation of E2F4/Rb complexes that bound to an E2F site in the cdc25A promoter (44). Taken together, these results demonstrated that the induced $p105^{Rb}$ and p130 proteins formed complexes with E2F family members and repressed a panel of E2F-regulated cell cycle regulatory genes.

These results suggest that the following sequence of events occurred in the Rb pathway (Fig. 6). Reduction in HPV18 E7 expression caused the stabilization of Rb family members, resulting in a posttranscriptional increase in their intracellular concentration. The increased concentration of $p105^{Rb}$ and p130 caused the assembly of E2F/Rb transcriptional repressor complexes, which bound to E2F sites located in the promoters of E2F-responsive genes required for cell cycle progression (45), resulting in their transcriptional repression. The repression of E2F-regulated genes also instituted positive feedback loops that

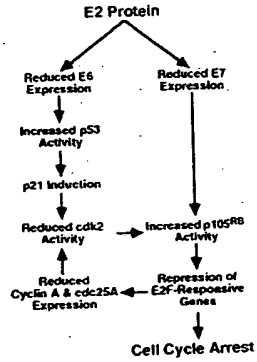

Fig. 6. Model for the growth regulatory pathway activated by the E2 protein. See text for details.

reinforced the growth inhibitory signal and ensured that growth inhibition was maintained. First, E2F1-mediated activation of genes required for S phase progression was reduced. Second, because E2F1 and p107 were absent from the cell, $p105^{Rb}$/E2F4 and p130/E2F4 complexes were more likely to form. Complexes containing these Rb and E2F family members have potent transcriptional repressor activity in nonproliferating cells (46–50). Finally, because both cyclin A and cdc25A stimulate cdk activity (51), their absence, together with the p53-mediated induction of p21, is predicted to impair cdk-mediated phosphorylation of $p105^{Rb}$ at the G_1/S boundary. The sequential induction of $p105^{Rb}$ followed by Rb-mediated repression of E2F-responsive genes that encode cdk activators may provide an explanation for the finding that levels of hypophosphorylated $p105^{Rb}$ rose before the reduction in the level of the hyperphosphorylated form.

Role of HPV E6/E7 Repression in Activation of the Rb Pathway. Francis *et al.* (41) previously reported that the E2-induced reduction in HeLa cell colony formation was impaired by constitutive expression of the HPV16 E6 and E7 genes, but cellular regulatory components were not examined in these experiments. Here, we assessed the effect of constitutively expressed HPV16 E6/E7 on the acute cellular and biochemical response to the E2 protein. All seven cell clones generated by infection with the empty retrovirus vector showed high level inhibition of DNA synthesis after introduction of the E2 gene, whereas the clones generated by infection with the HPV16 E6/E7 retrovirus displayed varying amounts of E2-resistant DNA synthesis (Fig. 7 *Top*; and R. DeFilippis & D.D., unpublished results). The incomplete protection of HeLa cells from the E2 protein may reflect suboptimal expression of HPV16 E6 and E7 from the heterologous promoter. A representative control cell clone and two clones generated by the HPV16 E6/E7 retrovirus were selected for biochemical analysis. As expected, expression of the E2 protein caused ≥99% reduction in the level of endogenous HPV18 mRNA (Table 1). In contrast, the transduced HPV16 E6/E7 genes were not repressed. E2-mediated induction of hypophosphorylated $p105^{Rb}$ and loss of hyperphosphorylated $p105^{Rb}$ were severely impaired in cells constitutively expressing HPV16 E6/7 compared with control cells (Fig. 7 *Middle*). In addition, E2-induced repression of cyclin A (Fig. 7 *Bottom*), and cdc25A (data not shown) was largely eliminated. We conclude that repression of HPV E6/E7 expression is required for E2-mediated induction of hypophosphorylated $p105^{Rb}$ and repression of E2F-responsive genes in HeLa cells.

Implications. These results have several important implications. First, E6/E7 repression clearly preceded growth inhibition and was required for efficient E2-induced growth inhibition and for acute activation of the Rb pathway and repression of E2F-responsive genes. In addition, the earliest biochemical changes we have detected in cell cycle components, the posttranscriptional increase in p53, $p105^{Rb}$, and p107 levels, can be simply explained by the loss of the E6 and E7 proteins, which otherwise target these tumor suppressor proteins for accelerated proteosome-mediated degradation. Thus, although numerous events drive the malignant conversion of cervical carcinoma cells, E6/E7 expression appears to be continuously required to maintain their proliferative state. Second, the response of the cell cycle machinery to E6/E7 repression is complex and dynamic, demonstrating that examination of cellular physiology at a single time point can be misleading. Third, the cellular response can be explained by the known regulatory circuits comprising the p53 and Rb tumor suppressor pathways, strongly suggesting that these two major tumor suppressor pathways are functionally intact in HeLa cells. Although these aneuploid cells express viral proteins that induce genetic instability, and they have accumulated numerous genetic aberrations during their progression to a malignant carcinoma, this underlying regulatory machinery is intact. Finally, the E2 protein reactivates these dormant tumor suppressor pathways in an orderly fashion, resulting in the transmission of multiple reinforcing signals that converge on the repression of E2F-responsive genes required for entry into S phase. Thus, a surprisingly simple

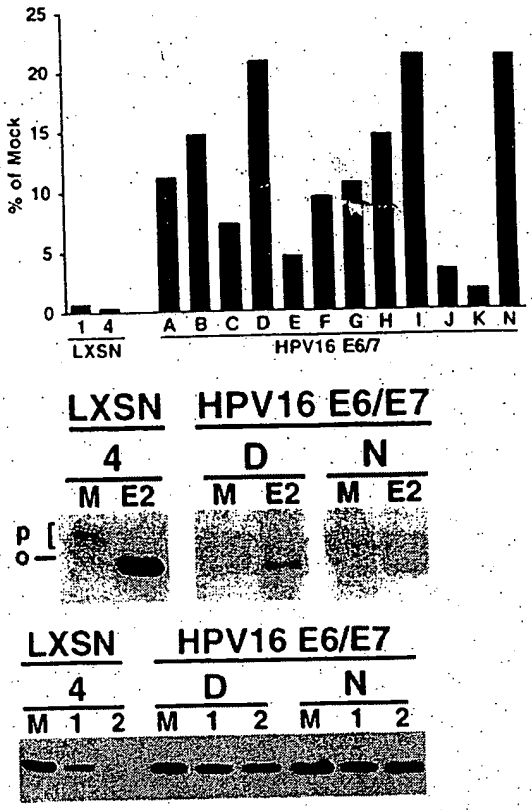

Fig. 7. Effect of HPV16 E6/E7 expression on the acute response to the E2 protein. Cell lines derived from individual clones of cells infected with control retrovirus (LXSN) or HPV16 E6/E7 retrovirus were analyzed. (*Top*) DNA synthesis by the indicated cell lines was measured by incorporation of tritiated thymidine 48 h after infection with the E2 virus, expressed as the percentage of DNA synthesis by each clone after mock-infection. (*Middle*) Expression of $p105^{Rb}$ in the indicated cell lines after mock-infection or 1 day after infection with the E2 virus. (*Bottom*) Expression of cyclin A in the indicated cell lines after mock-infection or 1 or 2 days after infection with the E2 virus.

Table 1. Repression of HPV18 mRNA in cells constitutively expressing HPV16 E6/7 mRNA

	% of mock*	
	HPV18 E6/E7	HPV16 E6/E7
LXSN-4	0.4	NA†
HPV16 E6/7-D	1.0	98.2
HPV16 E6/7-N	0.8	220

*HPV E6/7 mRNA prepared 24 h after infection with the E2 virus was measured by Northern blotting, normalized, and expressed as the percentage of the signal from mock-infected cells. The average of two different experiments is shown.

†NA, not applicable.

genetic manipulation is sufficient to mobilize this regulatory machinery and impose a cell cycle block.

Unlike most cancers, in which the brakes on cell growth are broken, in HeLa cells the driver is asleep. Expression of the E2 protein is sufficient to wake up the driver and impose growth control. Our results suggest that other manipulations that inhibit the expression or activity of the HPV E6 and E7 proteins will have a similar effect. Thus, the integrity of tumor suppressor pathways in cervical cancer cells may provide a unique target for therapy.

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